Toxicological effects of individual and mixtures of mycotoxins and persistent organochloride pesticides on reproduction using *in vitro* bioassays

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Abstract

There is a gradual decline in human reproductive health globally and environmental and food-borne toxins, including mycotoxins and persistent organochloride pesticides (POPs) have been suggested as one of the possible causes. However, the reproductive health effects of both individual and mixtures of these toxins are still poorly characterised. The application of suitable *in vitro* cellular models and relavant bioassays are critical to the understanding of these effects. The MMV-Luc, MA-10 Leydig and BeWo placental cell lines were used to evaluate the effects of individual and mixtures of selected mycotoxins and POPs on reproduction. In the reporter gene assay (RGA), zearalenone (ZEN) and α-zearalenol (α-ZOL) at biologically relevant concentrations induced oestrogen receptor (ER) transcriptional response (75% - 85%) relative to the response mediated by 10 nM of 17β-oestradiol (E_2) standard arbitrarily set at 100% response. Co-exposure of single mycotoxins with clinically relevant concentrations of E_2 (0.05 nM or 10 nM) had stimulatory or inhibitory effect on E_2-mediated ER transcriptional response depending on dose whereas mixtures of equimolar concentration (50 µM) of 1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethylene (*p,p'*-DDT) and 1, 1, 1-trichloro-2,2-bis (*p*-chlorophenyl)-ethane (*p,p'*-DDE) with E_2 at similar doses completely abolished ER response. Among single toxins, deoxynivalenol (DON) and ochratoxin A (OTA) were significantly cytotoxic to both MA-10 and BeWo cell lines. In the endocrine function, ZEN and its metabolites elevated progesterone (P4) production, but inhibited testosterone level in MA-10 cells whereas they had no effect on P4 levels in BeWo cells, but significantly increased E_2 production. All the tested mycotoxins and POPs significantly inhibited the production of beta-human chorionic gonadotropin (β-hCG) in BeWo placental cells. The effects of binary mycotoxin and POPs combinations on cell viability and hormone production showed synergistic, additive or antagonistic effects depending on the cell model, tested concentrations or mixture. In the quantitative real-time PCR (RT-qPCR) assay, ZEN, *p,p'*-DDT, and their combination significantly elevated the mRNA expression of genes of the insulin-like growth factor (IGF) axis (*IGF2BP1* and *IGF2R*), imprinted genes (*PHLDA2* and *MEG3*) and genes for DNA methylation (*MTHFR* and *MDB2*) in BeWo cells. Overall, this study provides the first comprehensive evidence that exposure to mycotoxins and/or POPs, individually or in combination, could have adverse effects on reproduction and development through multiple mechanisms.

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Abbreviations

4-OH-OTA	4-hydoxyl-ochratoxin A
ABCC1	ATP-binding cassette, sub-family C (CFTR/MRP), member 1
ABCC2	ATP-binding cassette, sub-family C (CFTR/MRP), member 2
AChE	Acetylcholinesterase
ACTB	Actin, beta
AF-alb	Aflatoxin-albumin adducts
AFB1	Aflatoxin B1
AFB2	Aflatoxin B2
AFG1	Aflatoxin G1
AFG2	Aflatoxin G2
AFL	Aflatoxicol
AFM1	Aflatoxin M1
AFM2	Aflatoxin M2
AFs	Aflatoxins
AhR	Aryl hydrocarbon receptor
AIDS	Acquired immunodeficiency diseases
ALP	Alkaline phosphatase
ANOVA	Analysis of variance
AOH	Alternariol
AP-1	Activator protein – 1
AR	Androgen receptor
ATCC	American type culture collection
ATSDR	Agency for Toxic Substances and Disease Registry

AZF	Azoospermia factor
b.w	Body weight
BHMT	Betaine--homocysteine S-methyltransferase
BLAST	Basic Local Alignment Search Tool
BLK	Blank
CALUX	Chemical activated luciferase gene expression
CAR	Constitutive androstance receptor
CAT	Catalase
CDH1	Cadherin 1, type 1, E-cadherin (epithelial)
cDNA	Complementary deoxyribonucleic acid
CI	Confidence interval
CIT	Citrinin
CONTAM	Contaminants in the Food Chain
CPP	Central precocious puberty
C_T	Cycle threshold
CYP11A1	Cytochrome P450, family 11, subfamily A, polypeptide 1
CYP17A1	Cytochrome P450, family 17, subfamily A, polypeptide 1
CYP19A1	Cytochrome P450, family 19, subfamily A, polypeptide 1
DH-CIT	Dihydrocitrinone
DHT	5α-dihydrotestoeterone
DMEM	Dulbeco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNMT1	DNA (cytosine-5-)-methyltransferase 1

DNMT3A	DNA (cytosine-5-)-methyltransferase 3 alpha
DNMT3B	DNA (cytosine-5-)-methyltransferase 3 beta
DOM-1	De-epoxy-deoxynivalenol
DON	Deoxynivalenol
DON-15-GlcA	Deoxyvalenol-15-O-glucuronide
DON-15-GlcA	Deoxynivalenol-15-glucuronide
DON-3-GlcA	Deoxynivalenol-3-glucuronide
DYSF	Dysferlin
E2	Oestradiol/17β-oestradiol
EC_{50}	Half maximal effective concentration
ECACC	European Collection of Authenticated Cell Cultures
EDCs	Endocrine disrupting chemicals
EDTA	Ethylenediaminetetraacetic acid
EFSA	European food safety authority
EGFP	Enhanced green fluorescent protein
ELISA	Enzyme-linked immunosorbent assay
ENNB	Enniatin B
EPT	12, 13 epoxytrichothec-9-ene
ER	Oestrogen receptor
ERVW-1	Endogenous retrovirus group W, member 1
ESR1/ERα	Oestrogen receptor alpha
ESR2/ERβ	Oestrogen receptor beta
FB1	Fumonisin B1
FB2	Fumonisin B2
FB3	Fumonisin B3

folbp1	Folate binding protein 1
FRα	Folate receptor alpha
FSH	Follicle stimulating hormone
FSN	School of Food Science and Nutrition, University of Leeds
FUMS	Fumonisins
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GD	Gestational day
GnRH	Gonadotropin-releasing hormone
GPER1	G-protein-coupled oestrogen receptor 1
GPR54	G-protein-coupled receptor 54
GPX	Glutathione peroxidase
GRX	Glutathione reductase
GSH	Glutathione
GST	Glutathione transferase
H_2SO_4	Tetraoxosulphate (VI) acid
HAZ	Height-for-age Z-score
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
HPRT1	Hypoxanthine guanine phosphoribosyl transferase 1
HSD17B1	Hydroxysteroid (17-beta) dehydrogenase 1
HSD3B1	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1

IARC	International Agency for Research on Cancer
IC_{50}	Half maximal inhibitory concentration
IGF1	Insulin-like growth factor 1 (somatomedin C)
IGF1R	Insulin-like growth factor 1 receptor
IGF2	Insulin-like growth factor 2
IGF2BP1	Insulin-like growth factor 2 mRNA binding protein 1
IGF2BP2	Insulin-like growth factor 2 mRNA binding protein 2
IGF2BP3	Insulin-like growth factor 2 mRNA binding protein 3
IGF2R	Insulin-like growth factor 2 receptor
INS	Insulin
JECFA	Joint FAO/WHO Expert Committee on Food Additives
KISS1	Kisspeptin
LacZ	Beta-galactosidase
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LEP	Leptin
LHCGR	Luteinizing hormone/choriogonadotropin receptor
LICAMM	Leeds Institute of Cardiocvascular and Metabolic Medicine, University of Leeds
Luc	Luciferase
MAPK	Mitogen-activated protein kinase
MBD2	Methyl-CpG binding domain protein 2
MDGs	Millennium Development Goals

MEG3	Maternally expressed 3 (non-protein coding)
MMP2	Matrix metalloproteinase 2
mRNA	Messenger ribonucleic acid
MTHFR	Methylenetetrahydrofolate reductase
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-dipenyltetrazolium bromide solution
NEO	Neosolaniol
NIV	Nivalenol
NOAEL	No-observed-adverse-effect-level
NSB	Non-specific binding wells
NTDs	Neural tube defects
o,p′-DDT	1, 1, 1-trichloro-2-(*0*- chlorophenyl)-2-(*p*-chlorophenyl)-ethane
OR	Odd ratio
OTA	Ochratoxin A
OTα	Ochratoxin alpha
p,p′-DDE	1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethylene
p,p′-DDT	1, 1, 1-trichloro-2,2-bis (*p*-chlorophenyl)-ethane
P4	Progesterone
PBS	Dulbecco's phosphate buffered saline
PCR	Polymerase chain reaction
PGK1	Phosphoglycerate kinase 1
PGR	Progesterone receptor
PHLDA2	Pleckstrin homology-like domain, family A, member 2
PPM	Parts per million

PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
PXR	Pregnane X receptor
RGA	Reporter gene assay
RH	Reproductive health
RLUs	Relative light units
RNA	Ribonucleic acid
RPLP0	Ribosomal protein, large, P0
RPM	Revolution per minute
RT-qPCR	Quantitative real-time polymerase chain reaction
Sa	Sphinganine
SD	Standard deviation
SDGs	Sustainable development goal
SDS	Sodium dodecyl sulfate
SEM	Standard error of mean
SHBG	Sex hormone-binding globulin
So	Sphingosine
SOD	Superoxide dismutase
StAR	Steroid acute regulatory protein
StAR	Steroidogenic acute regulatory protein
TAA	Total ascorbic acid
TCA	Tricarballylic acid
TET3	Tet methylcytosine dioxygenase 3
TMB	3,3’,5,5’-Tetramethylbenzidine
TNF-α	Tumour necrosis factor-alpha
U2OS	Osteosarcoma cells

UPH	Ultra-high purity water
VEGFA	Vascular endothelial growth factor A
WAZ	Weight-for-height Z-score
WHO	World Health Organisation
yEGFP	Yeast enhanced green fluorescent protein
YWHAZ	Tyrosine 3-monoxygensase/tryptophan 5-monoxygenase activation protein, zeta
ZAN	Zearalanone
ZEN	Zearalenone
ZEN-14-GlcA	Zearalenone-14-O-glucuronide
ZEN-14-GlcA	Zeralenone-14-O-glucuronide
ZFP57	Zinc finger protein 57
α-ZAL	α-zearalanol
α-ZOL	α-zearalenol
α-ZOL-14-GlcA	α-Zearalenol-14-O-glucuronide
α-ZOL-7-GlcA	α-Zearalenol-7-O-glucuronide
β-hCG	Beta-human chorionic gonadotrophin
β-ZAL	β-zearalanol
β-ZOL	β-zearalenol
β-ZOL-14-GlcA	β-Zearalenol-14-O-glucuronide
β-ZOL-16-GlcA	β-Zearalenol-16-O-glucuronide
γGT	γ-glutamyltransferase

Chapter 1

1. General introduction and literature review

1.1 Background of study

Reproduction is the biological process through which organisms bring forth an offspring of their kind. This is fundamental to all life. Human reproductive health (RH) has been defined as '*a state of complete physical, mental and social well-being and not merely the absence of diseases or infirmity of the reproductive system, including its functions and processes*' (WHO, 1999). It encompasses all the reproductive processes, functions and systems at all stages of life, and includes fertility, maternal health, *in utero* development, and newborn and child health. Due to its impacts on the general population health, RH is a crucial feature of healthy social, economic and global development. Unfortunately, human reproductive health has increasingly deteriorated over recent years and has been estimated to account for about 20% of the global burden of disease (WHO, 1999). This was highlighted in the United Nations Millennium Development Goals (MDGs) which ended in 2015, an international framework for assessing progress towards promoting global development and eliminating poverty (United Nations, 2000). Out of the eight MDGs, four were directly related to the principles of reproductive health (Goals 3 - 6: promote gender equality and empower women; reducing child mortality; improving maternal health; and combating HIV/AIDS, malaria, and other diseases). Due to the failure to achieve the desired goals, three of the four goals which sit on reproductive health were incorporated in goal 3 (good health and well-being) of the seventeen sustainable development goals (SDGs) set by United Nations General Assembly in 2015 (United Nations, 2015). The reproductive health status of an individual can be influenced by several factors such as genetic susceptibility, behavioural factors, infectious diseases, and other health indices. However, environmental factors have been suggested as possible contributing factors to poor reproductive health. For instance, food toxins (e.g. mycotoxins) and environmental contaminants (e.g. pesticides and pharmaceutical products) have been suggested to cause adverse effects on reproduction in both human and wildlife (Ibe et al. 1994; Skakkebæk et al., 2001; Connolly et al. 2011; Martenies and Perry 2013), but the mechanisms remain unclear and demands further exploration.

Some of these food-borne toxins and environmental contaminants are classified as endocrine disrupting chemicals (EDCs). Several definitions of an EDC have been put forward by different organisations or researchers over the years and therefore, the definition has evolved over time. According to the European Union (EU), an EDC is an '*exogenous substance that causes adverse health effects in an intact organism, or its progeny, secondary to changes in endocrine function*' (IPCS, 2002). On the other hand, the US Environmental Protection Agency (EPA) defined an EDC as '*an exogenous agent that interferes with the synthesis, secretion, transport, metabolism, binding action, or elimination of natural blood-borne hormones that are present and are responsible for homeostasis, reproduction, and development process*' (Diamanti-Kandarakis et al., 2009). Arguably, the most currently accepted definition is the one put forward by the Endocrine Society which states that an EDC is '*an exogenous chemical, or mixtures of chemicals, that interferes with any aspect of hormone action*' (Zoeller et al., 2012). These EDCs potentially affect reproduction, development, and/or behaviour by interfering with normal synthesis, storage, release, transport, metabolism, binding, action or elimination of endogenous hormones (Diamanti-Kandarakis et al., 2009). The interference of hormonal homeostasis is thought to be mediated through the following mechanisms (i) mimic the effect of endogenous hormones; (ii) antagonise the effect of endogenous hormones; (ii) disrupt the synthesis and metabolism of endogenous hormones; (iv) disrupt the synthesis of hormone receptors; or (v) alter target cell sensitivity (Mendes et al., 2002; Diamanti-Kandarakis et al., 2009). Most studies evaluating the endocrine disrupting effects of environmental and food contaminants have focused on their adverse impacts on steroidogenesis due to the central role sex steroid hormones play in reproduction and development. Steroid hormones are useful for the proper development and function of the reproductive organs in both humans and animals. In females, oestrogen (E2) and progesterone (P4) supports ovarian function such as follicular development, oocyte maturation and regulation of ovulation as well as in embryo implantation, placental function and foetal development (Cross, 2006; Guibourdenche et al., 2009; Prouillac and Lecoeur, 2010). In males, testosterone and 5α-dihydrotestoeterone (DHT) are the major hormones involved in the regulation of spermatogenesis and sexual differentiation (Akingbemi, 2005). However, reports also indicate that oestrogen is involved in spermatogenesis through stimulation of germ cell proliferation, differentiation and the final maturation of spermatids, as well as germ cell survival and apoptosis (Carreau and Hess, 2010). The production of steroid hormones

(steroidogenesis) involves the mobilisation of cholesterol to the inner mitochondrial membrane by steroid acute regulatory protein (StAR). Then cholesterol is converted to progestagens (e.g. pregnenolone and progesterone), followed by further metabolism to androgens (e.g. testosterone & DHT) and oestrogens including estrone and 17β-estradiol (**Fig. 1.1**). The negative effects of mycotoxins on the reproductive system and development have been reported in different animal models and primary cells *in vitro* (**see section 1.2**). The most studied mycotoxin with regard to endocrine disruption is zearalenone (ZEN) and its metabolites as a result of their ability to interfere with oestrogen receptor (ER) activity and steroidogenesis *in vivo* and *in vitro* (Connolly et al., 2011). The pesticide 1, 1, 1-trichloro-2,2-bis (*p*-chlorophenyl)-ethane (*p,p'*-DDT) and its major metabolite 1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethylene (*p,p'*-DDE) are both ER agonists, and *p,p'*-DDE is also an androgen receptor (AR) antagonist (Kelce et al., 1995) and both causes perturbation in steroid hormone production (ATSDR, 2002).

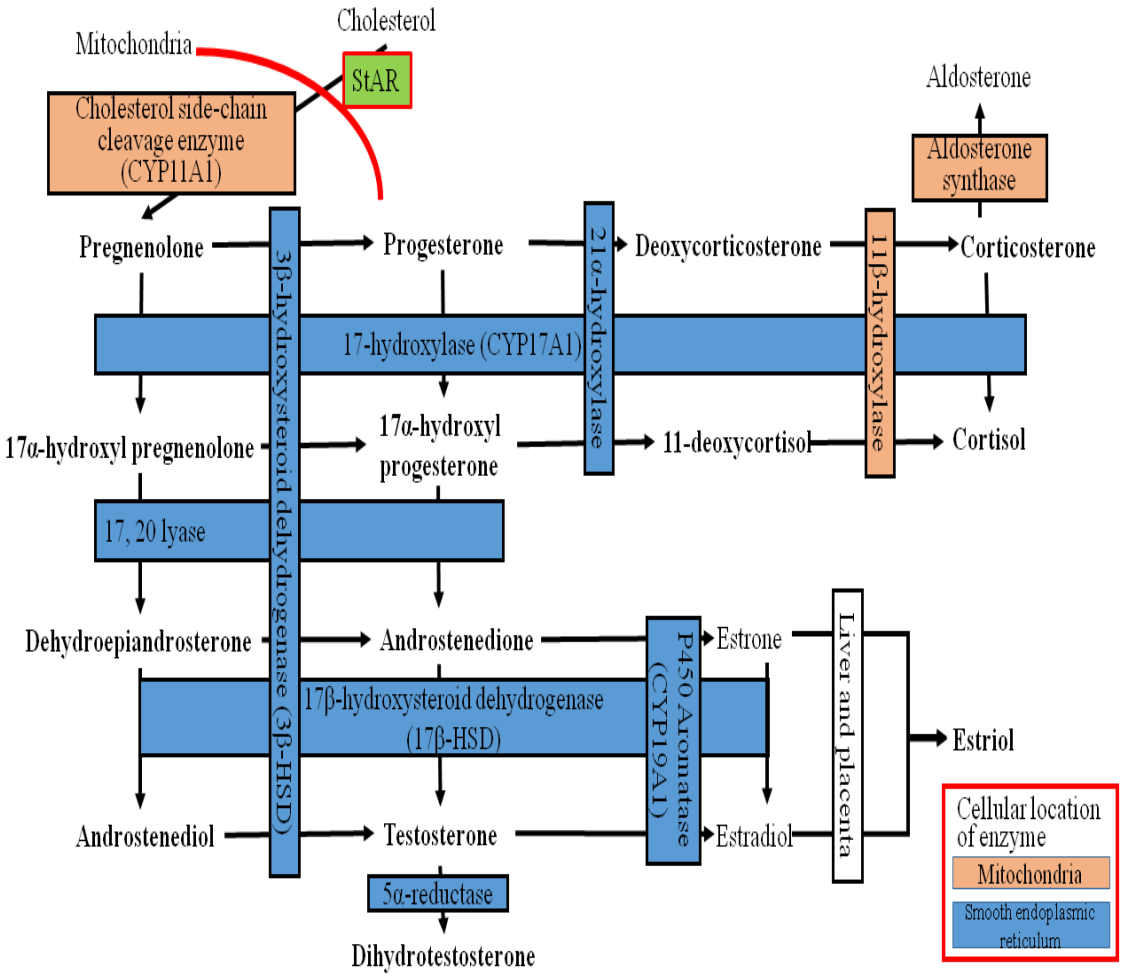

Figure 1.1: The steroidogenesis pathway showing hormones and metabolic enzymes

Apart from endocrine-related effects, mycotoxins and environmental pollutants are also known to cause functional and morphological alterations in reproductive organs and sex gametes. For instance, ZEN was suggested as the cause of ovarian follicle atresia, whereas ZEN and deoxynivalenol (DON) induced meiotic chromatin degeneration of oocytes, and causes aneuploidy and abnormal embryo development in farm animals (Malekinejad et al., 2007; Minervini and Dell' Aquila, 2008). In addition, ZEN causes deoxyribonucleic acid (DNA) fragmentation and testicular germ cell apoptosis in rats (Kim et al., 2003) and induced boar sperm DNA damage after exposure to different concentrations of ZEN *in vitro* (Tsakmakidis et al., 2008). AFB_1, ZEN and metabolites, DON and OTA as well as *p,p'*-DDT and its metabolites have been reported to pass through the placenta and can negatively affect embryo and foetal development *in utero* (Wild et al., 1991; Rogan and Chen, 2005; Nielsen et al., 2011; Woo et al. 2012; Minervini et al., 2013) which may result in adverse reproductive health outcomes. ZEN and its metabolites have also been identified in the follicular fluids of pigs (Sambuu et al. 2011) and cattle (Takagi et al. 2008) emphasising the need for research into their adverse effects on reproduction and foetal development. For instance, placental transfer of ZEN and its metabolites have been reported in pregnant sows and mice reducing the percentage of normal primordial follicles and causing anomalies in foetal testicular development, respectively (Perez-Martinez et al., 1996; Schoevers, et al., 2012). Furthermore, ZEN reduced the quantity of healthy follicles in the F-1 prepubertal gilts after pregnant sows were exposed to feeds contaminated with ZEN (200, 500 and 1000 μg/kg feed) and this may result in premature oocyte depletion in adulthood (Schoevers, et al., 2012). In porcine oocytes exposed to DON *in vitro*, it was demonstrated that DON inhibited the maturation and disrupted meiotic spindle formation of the oocytes resulting to the retardation of cell cycle progression (Han et al., 2016). High incidence of sperm DNA damage is also weakly associated with *p,p'*-DDT exposure (De Jager et al., 2009). This is of great concern as sperm DNA damage has been associated with infertility, poor embryo quality, spontaneous abortion and genetic abnormalities in the newborn (Stocks et al., 2010; Lewis et al., 2013). The mechanisms through which xenobiotics cause sperm DNA damage is still unclear, however, oxidative damage and aberrant apoptosis in exposed cells have been the common observations (Lewis and Aitken, 2005; Pacey, 2010).

Exposures to different environmental and food contaminants during the critical periods of human development may also influence reproductive health status through the process of epigenetics. The term epigenetics refers to phenotypic or heritable alterations in the gene expression that are not caused by changes in DNA sequence (Zhou et al., 2014). Epigenetic modifications, including DNA methylation, histone modifications and micro RNA, lead to the induction or repression of gene expression and they are inherited by both mitotic and meiotic patterns (Portela and Esteller, 2010). In mammals, DNA methylation usually occurs at the 5'-position of the pyrimidine ring of the cytosine residues and involves the transfer of a methyl group (-CH3) from S-adenosyl methionine to the fifth carbon atom of the cytosine ring to form 5-methylcytosines in the cytosine-phosphate-guanine (CpG) dinucleotides (Aliberti and Barile, 2014). The DNA methyltransferases (DNMTs), including DNMT1, DNMT3A and DNMT3B are responsible for the methylation of DNA and uses S-adenosyl-methionine as the methyl donor (Portela and Esteller, 2010). The DNMT1 is the major maintenance methyltransferase and has strong preference for the hemimethylated CpG dinucleotides in the genome during DNA replication or cell division. The *de novo* DNMTS (DNMT3A and DNMT3B) methylate both previously unmethylated and hemimethyated DNA, and their activity is independent of DNA replication (Jenkins and Carrell, 2011). The hypermethylation of CpG islands inhibits transcription whereas hypomethylation of the CpG islands induces transcription by allowing interaction for transcription factors (Portela and Esteller, 2010). The genome of mammals undergoes reprogramming in DNA methylation patterns, especially in the primordial germ cells and during early pre-implantation embryos, and the epigenome is susceptible to changes induced by xenobiotics during these critical stages of development thereby potentially predisposing the child to adverse health effects in later life (Reik et al., 2001). These epigenetic events are critical for cell division and proliferation as well as normal germline and embryo functions (Robertson and Wolffe, 2000). A detailed review of DNA methylation and the mechanisms of epigenetic inheritance has previously been performed by Aliberti and Barile, (2014).

It has been demonstrated that DON disrupted epigenetic modification of oocytes by increasing the level of DNA methylation and altering *DNMT3A* mRNA levels in treated cells (Han et al., 2016). Similarly, ZEN exposure also reduced the developmental competence of matured mouse oocytes through the increase of DNA methylation in the

exposed oocytes (Zhou et al., 2014). Taken together, it can be deduced that these mycotoxins and environmental contaminants have the potential to adversely affect ovarian and placental function as well as foetal development. However, further study is required to elucidate the molecular pathways responsible for these effects.

1.2 Mycotoxin exposure and adverse reproductive health outcomes

Mycotoxins are biologically active secondary metabolites naturally produced by multiple species of fungi (*Aspergillus*, *Penicillium*, and *Fusarium*) during their growth on food crops either in the field, during harvest or storage (Pitt et al., 2013). Mycotoxins contaminate many staple food crops and crops used in animal feed, particularly in sub-Saharan Africa and Asia (Pitt et al., 2013). Over 400 chemically diverse mycotoxins have been identified, however, those that are of public health significance include aflatoxins (AFs), ochratoxin A (OTA), fumonisins (FUMS), zearalenone (ZEN) and deoxynivalenol (DON) due to their frequent occurrence in agricultural products and their adverse health effects in humans (Schatzmayer and Streit, 2013). Exposure tends to be highest in low income communities where food may be grown and stored locally, where there is a low diversity in diet and where socioeconomic deprivation contributes to malnutrition (Wild and Gong, 2010). A number of adverse health effects have been reported in various mycotoxins, with varying degrees of evidence. In regions where mycotoxin contamination of food is often high, there are often also high levels of reproductive health problems in humans such as high rates of infertility, low birth weight, pre-term delivery, still births and birth abnormalities (McClure et al., 2009; Mascarenhas et al., 2012; Ota et al., 2014; Agarwal et al., 2015). Causes of these reproductive health problems are multifactorial and undernutrition and poverty are major contributors. Nevertheless, the occurrence of chronic mycotoxin exposure in human populations affected by high rates of such reproductive health problems has led to research into the possible role of mycotoxins as causes of these conditions. Most human research on the adverse reproductive health effects of mycotoxins has focused on AFs, FUMS and ZEN, but there are also several animal and *in vitro* studies reporting reproductive toxicity and endocrine disrupting effects of other mycotoxins, including DON, OTA, nivalenol (NIV), and T-2 toxin. Therefore, this review is focused on the evidence that mycotoxins contribute to a range of human reproductive health

conditions, with aim of guiding research designed to understand mechanisms through which mycotoxins cause adverse reproductive health outcomes.

1.2.1 Search methods

For the review, all relevant data were obtained through PubMed, MEDLINE, EMBASE, Scopus, Google Scholar and Web of Science databases for all English language articles published from January, 1979 to June, 2017. The search method followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines for the reporting of systematic reviews (Moher et al., 2009). Search terms and concepts used include the following: infertility, birth outcomes, foetal health, reproduction, reproductive health, reproductive organs, reproductive performance, pregnancy, preterm birth, birth defects, neural tube defects, foetal loss, low birthweight, small for gestational age, miscarriage, foetal deaths, stillbirths, maternal anaemia, newborns, neonatal health, precocious puberty, premature thelarche, combined with mycotoxins, aflatoxins, fumonisins, zearalenone, deoxynivalenol, nivalenol, or ochratoxin A in the search. Journals were also hand-searched and reference lists of published articles were scanned for potential eligible studies. In addition, titles of identified articles were screened to futher evaluate their eligibility for inclusion in the review. A total of 100 potential studies were identified. Only 24 studies with outcomes relating to mycotoxin exposure and human reproductive or developmental health were retained in the review.

1.2.2 Aflatoxins

Aflatoxins (AFs) are major metabolites of *Aspergillus flavus* and *Aspergillus parasiticus* and are frequent contaminants of peanuts, maize, rice, and oil seeds, particularly in Africa and South Asia (IARC, 2002). The naturally occurring classes of AFs are AFB_1, AFB_2, AFG_1 and AFG_2 (**Fig. 1.2**) and the hydroxylated forms commonly found in milk and urine (AFM_1 and AFM_2) (Kensler et al., 2011). Aflatoxin B_1 (AFB_1) is the most toxic and is a major risk factor for hepatocellular carcinoma, particularly in individuals with chronic hepatitis B virus infection (IARC, 2002; Kensler et al., 2011). Acute toxicity causes liver failure and can be fatal. AFB_1 has also been associated with growth impairment in children and modification of immune function (IARC, 2015). In

addition, recent evidence indicates AFs may be involved in adverse reproductive health outcomes (Shuaib et al., 2010a; Smith et al., 2017a).

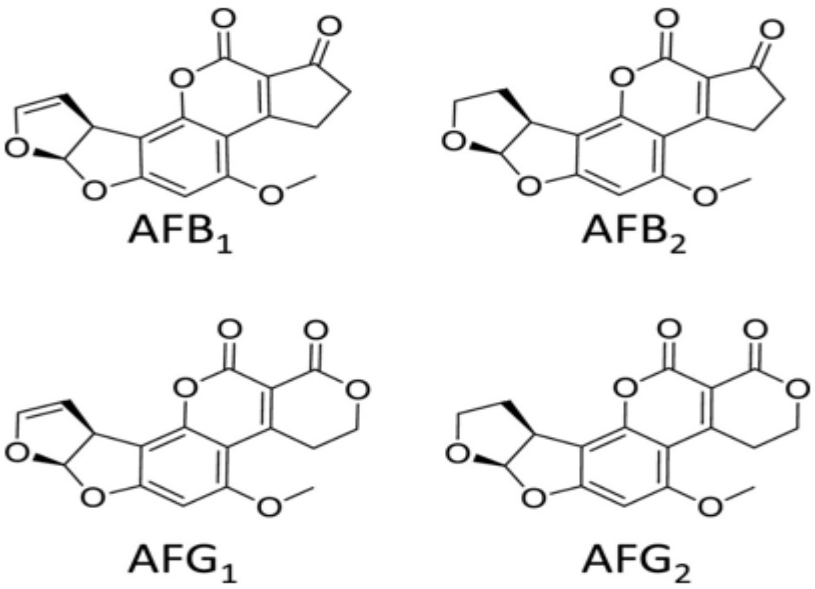

Fig. 1.2: Chemical structures of the major aflatoxins found in food (Source: McMillan et al., 2018; Reproduced with permission obtained from the publisher Elsevier)

1.2.2.1 Maternal aflatoxin exposure and adverse pregnancy outcomes

AFs and aflatoxin-albumin (AF-alb) adducts have been detected in cord blood samples indicating that AFB_1 crosses the placental barrier reaching foetal circulation (Denning et al., 1990; Wild et al., 1991). AF exposure in pregnant women is mostly due to ingestion of contaminated diets, and has been suggested to contribute to poor maternal, neonatal and child health, especially in Africa and Asia (Shuaib et al., 2010a). The consequences of AF exposure in mothers, neonates and children are many and they include anaemia in pregnancy, intrauterine growth restriction and low birth weight, interference with nutrient absorption, suppression of immune function, child growth retardation and abnormal liver function (IARC, 2015).

1.2.2.1.1 Aflatoxin exposure during pregnancy, intrauterine growth restriction and low birth weight

Maternal AF exposure during pregnancy has been associated with poor maternal, neonatal and child health in Africa (**Table 1.1**). Studies evaluating aflatoxin exposure

through the presence of aflatoxins or metabolites in serum (AFB_1, AFB_2, AFG_1 and AFG_2) or milk (AFM_1 and AFM_2), instead of validated AF exposure biomarkers (urinary AFM_1 and AFB_1-N7-guanine adduct or serum AF-alb adduct), reported associations between exposure and low birth weight (De Vries et al., 1989), still birth (Lamplugh et al., 1988) and neonatal jaundice (Ahmed et al., 1995; Abulu et al., 1997). In the Gambia, AF-alb adduct levels during pregnancy were associated with lower height-for-age (-0.207 *z* scores, $P = 0.044$) and lower weight-for-age (-0.249 *z* scores, $P = 0.012$) in infants during the first year of life (Turner et al., 2007). Shuaib et al. (2010c) also reported that of 755 pregnant Ghanaian women, those in the highest quartile of AF-alb (AF-alb: > 11.34pg/mg albumin) were more likely to have babies with low birth weight (OR, 2.09; 95% CI, 1.19–3.68) compared to the lowest quartile (AF-alb: ≤ 2.67pg/mg albumin). Mycotoxin exposure biomarkers have advantages over exposure assessments through food and/or foodstuff as they provide more objective data on mycotoxins exposure, and correct for heterogeneous distribution of the mycotoxins in food. Validated biomarkers of AF exposure are biomarkers shown in epidemiological studies to be quantitatively associated with dietary AF intake and include urinary AFM_1 and AFB_1-N7-guanine adduct as well as serum AF-alb adduct (Kensler et al., 2011). Urinary AFM_1 and AFB_1-N7-guanine adduct reflect AF exposure in the previous 24 – 48 hours while serum AF-alb adduct identifies AF exposure over the previous 2 – 3 months, and are therefore applied to detect short-term and long-term exposure, respectively. Several reports have shown a significant correlation between serum AF-alb adduct and urinary AFM_1 levels (Jolly et al., 2006; Piekkola et al., 2012; Chen et al., 2017) and therefore, reports involving these biomarkers should be accepted with confidence.

One postulated mechanism by which AF exposure could impact on growth is through interference with the insulin-like growth factor (IGF) axis. In Kenyan children exposed to aflatoxins *in utero*, reduced insulin growth factor 1 (IGF1) mRNA and protein levels were observed (Castelino et al., 2015). *In vitro* exposure of human hepatocyte cell line 16 (HHL-16) to AFB_1 also significantly decreased IGF1 mRNA and protein expression (Castelino et al., 2015). Furthermore, differential DNA methylation of fibroblast growth factor-12 (*FGF12*) and insulin growth factor 1 receptor (*IGF1*R) genes were observed in the white blood cells from Gambian infants whose mothers were exposed to aflatoxins during early pregnancy (Hernandez-Vargas et al., 2015). *IGF1* and *IGF1R* genes are

mediators of growth hormone and play major role in embryonic development, foetal and post-natal growth (Baker et al., 1993). It has been reported that serum IGF1 levels are significantly reduced in infants with intrauterine growth restriction compared to controls (Leger et al., 1996). Although these studies provide an insight into the negative effects of aflatoxins on the IGF1 axis leading to perinatal and neonatal growth restriction and low birth weight, these mechanisms remain unclear and need to be further explored to demonstrate causal association. As AFB_1 is known to cross the placental barrier, it is also possible that foetal cytochrome (CYP) 3A7 bioactivates AFB_1 to the toxic AFB_1-8, 9-epoxide after placental transfer of AFB_1 from maternal blood during pregnancy resulting in foetal toxicity (Partanen et al., 2010).

1.2.2.1.2 Aflatoxin exposure in pregnancy and maternal anaemia

Anaemia in pregnancy is one of the leading causes of maternal mortality during child birth in Africa and Asia (Khan et al., 2006; IFPRI, 2016). Recent studies assessing AF-alb adduct levels in pregnant women in Ghana reported significant association between AF exposure and anaemia in pregnancy as well as low birth weight. Shuaib et al. (2010b) reported that AF-alb level was associated with anaemia in pregnancy with the odds of being anaemic increasing by 21% (OR, 1.21, p=0.01) and each quartile of AF-alb reaching an 85% increased odds in the highest quartile (AF-alb: > 11.34pg/mg albumin) compared to the low quartile (AF-alb: ≤ 2.67pg/mg albumin) group (OR, 1.85; CI, 1.16-2.95).

Experimental studies have shown that AF promotes the haemolysis of erythrocytes, inhibits haematopoiesis, impairs iron absorption, affects haemoglobin levels and induces microcytic hypochromic anaemia in multiple animal species (Lanza et al., 1978; Verma and Raval, 1991; Kumar and Balachandran, 2005; Andretta et al., 2012; Yousef et al., 2003; Eisa et al., 2011). Lanza et al. (1978) explored the relationship of iron absorption to the develpment of anaemia associated with anaemia. Lanza and colleagues (Lanza et al., 1978) exposed 64 chicks to 5 μg/kg of AF b.w/day. It was observed that AF treatment caused reduction in body weight of the exposed chicks and decreased iron absorption (Lanza et al., 1978). In a similar study, Yousef et al. (2013) fed 15 rabbits with different concentrations of AF (15 μg/kg bw and 30 μg/kg bw). It was demonstrated that AF decreased erythrocyte count, haemoglobin concentration and

packed cell volume in the treated rabbits compared to untreated controls. In addition, microcytic hypochromic anaemia was also found in the treated rabbit group (Yousef et al., 2013). In a more recent meta-analyses of 98 studies containing 37,371 broilers which were fed 0 - 5 mg/kg of AF for an average of 18 days, Andretta et al. (2012) reported a reduction in packed cell volume, haemoglobin level and leukocyte count in AF-treated broilers. However, it must be emphasised that the concentrations of AF used in the above studies are higher than levels found in human epidemiological studies. It has also been hypothesised that AF could cause anaemia by impairing iron absorption and bioavailability as well as inhibition of erythropoiesis (Smith et al., 2017a). To date, no human research has been performed to test this hypothesis.

1.2.2.2 Aflatoxin exposure and male infertility

Male infertility is increasing in both developed and developing countries (Mascarenhas et al., 2012). The prevalence of male infertility varies between developed countries with USA standing at 6% (Chandra et al., 2013) whereas it is higher in UK at 10.1 – 12.5% in UK (Datta et al., 2016). In sub-saharan Africa, infertility prevalence rates are higher and range from 20-35% (Etuk, 2009). The 'infertility belt' stretching from West Africa, through Central to East Africa are known geographical regions with high infertility prevalence in Africa (Etuk, 2009). A previous case control study has pointed to environmental and food-borne contaminants (e.g. pesticides, phthalates, bisphenol A, mycotoxins, cadmium, lead, arsenic, and caffeine) as possible causes of male infertility in Africa (Okonofua et al., 2005), but the exact contaminants have yet to be identified. Subsequently, it has been argued for further research to uncover the role of food contaminants, such as AFs in the rising incidence and prevalence of male infertility in the African region (Eze and Okonofua, 2015) as these toxins are major contaminants of local foods consumed in the region. Although preliminary experiments have identified possible roles for AFs in the causation of infertility in the animal models (Ortatatli et al., 2002; Agnes et al., 2003; Ahmed et al., 2012; Supriya et al., 2014), this is yet to be confirmed in humans, where little research is available.

There are a few human studies reporting the potential of AF exposure in causing male infertility in Africa (**Table 1.1**). In Nigeria, Ibeh et al. (1994) examined the presence of

AFs in the semen of 100 adult males consisting of 50 infertile men and 50 fertile men to ascertain if there is a relationship between AFs and male infertility. Among the 50 semen samples from the infertile group, 20 (40%) had detectable level of AFs and 50% of the semen samples had sperm with abnormalities in sperm count, morphology and motility whereas only 4 (8%) of the fertile males had AFs in their semen with 10-15% of the semen showing sperm abnormalities. In addition, the concentrations of AFs found in the semen of infertile males were higher than fertile males. When adult male albino rats were fed with animal feed contaminated with 8.5 µg AFB_1 per gramme of feed for 14 days, they had decreased sperm count, abnormal morphology, reduced motility and viability (Ibeh et al., 1994). In an *in vitro* study, AFs also significantly reduced the motility of sperm cells (Ibeh et al., 2000). In a case-control study involving 30 infertile and 25 fertile males, Uriah et al. (2001) found significantly higher levels of AFB_1 in the blood (700 – 1392 ng/mL) and semen (60 - l48 ng/mL) of the infertile men compared to the fertile men. A case-control hospital-based study that examined the AF concentrations in semen samples from 108 men (60 infertile men and 48 fertile controls) visiting a University Hospital in Egypt has also been reported (Mohammed et al., 2014). It was observed that AFB_1 was present in 25% of the semen of infertile patients, compared to 2.1% found in fertile controls ($p = 0.0007$). Among the infertile patients with AFB_1 exposure, severe reduction in sperm count, reduced sperm motility, high percentage of sperm with abnormal morphology, and high viscosity in the semen were evident compared to the fertile group with low detectable AFB_1 (Mohammed et al., 2014).

The negative effects of AFB_1 on sperm parameters could be attributed to its ability to interfere with the biosynthesis of androgens and alteration of testicular function resulting in the inhibition of spermatogenesis and sperm maturation giving rise to the production of abnormal sperm cells (Picha et al., 1986; Agnes and Akbarsha., 2003; Ahmed et al., 2012; Adedara et al., 2014; Supriya et al., 2014). For instance, in adult male roosters fed with diets containing AFB_1 (5, 10 and 20 mg/L) for 8 weeks, a suppression of spermatogenesis, abnormalities in sperm morphology and count, reduced plasma testosterone and testicular atrophy were observed compared to the control adult roosters (Ortatatli et al., 2002). In a more recent study, exposure of bull semen to AFB_1 (0.1, 1, 10 and 100 µM) *in vitro* for 4 hours decreased sperm viability, caused hyper-

polarization of the sperm mitochondrial membrane and negatively affected sperm DNA integrity (Komsky-Elbaz et al., 2018).

1.2.3 Fumonisins

Fumonisins are primarily produced by *Fusarium verticillioides* (previously known as *Fusarium moniliforme*) and *Fusarium proliferatum* which contaminate maize and maize-based food products, although their presence in other agricultural products (e.g. sorghum, cowpea, asparagus, rice and farro) has been occasionally reported (Voss et al., 2006; Bulder et al., 2012; Rheeder et al., 2016). Human exposure to fumonisin B_1, B_2, B_3 and B_4 (**Fig. 1.3**) is high in regions where home-grown maize is used as a dietary staple (Gong et al., 2008; van der Westhuizen et al., 2011). Fumonisin B_1 (FB_1) is the most common of the numerous fumonisin analogues and the most studied fumonisins, and has been classified as a possible human carcinogen (Group 2B) by the International Agency for Research on Cancer (IARC, 2002).

Consumption of FB_1-contaminated maize and maize-based foods has been associated with a high occurrence of oesophageal cancer in the former Transkei region of South Africa (Rheeder et al., 1992; Misihairabgwi et al., 2017) and Linxian region of China (Sun et al., 2007). Similarly, in Mazandaran Province of Iran where there is also a high incidence of oesophageal cancer, increased concentrations of fumonisins have been detected in maize grown in the area (Shephard et al., 2002; Yazdanapanah et al., 2006; Alizadeh et al., 2012). In animals, FB_1 has been linked to spontaneous equine leukoencephalomalacia (Marasas et al., 1988; Smith et al., 2002), porcine pulmonary oedema (Harrison et al., 1990; Hascheck et al., 2001), liver and kidney toxicity in different animal species (Voss et al., 2001; Bolger et al., 2001), and liver and kidney cancer in rodents (Gelderblom et al., 1991; Howard et al., 2001a,b).

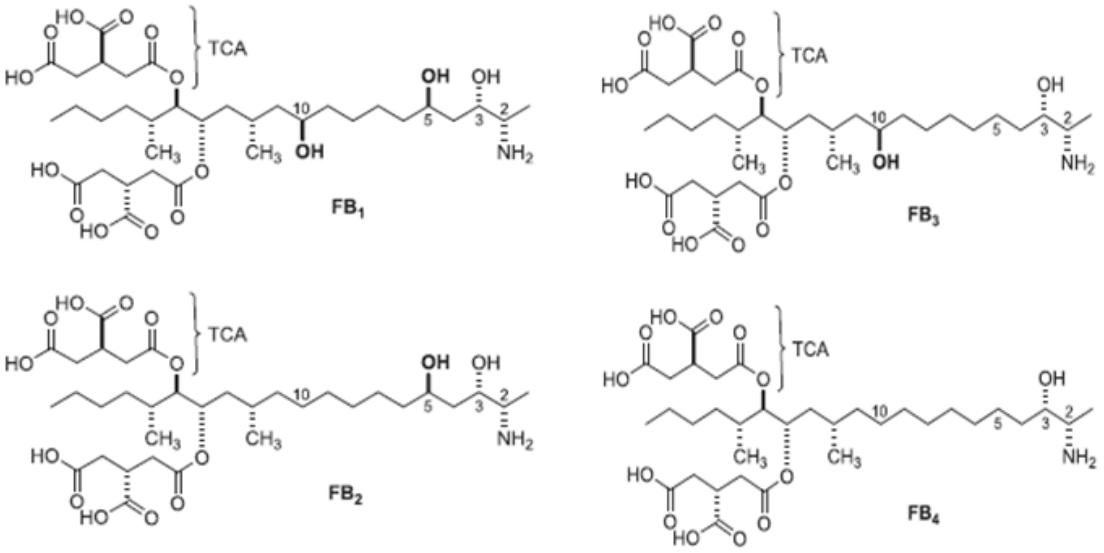

Fig. 1.3: Chemical structures of fumonisins. TCA: tricarballylic acid (Source: EFSA, 2018)

1.2.3.1 Fumonisin exposure, reproductive toxicity and birth defects

Growth retardation, delayed or incomplete ossifications, cleft palate or hydrocephalus, and foetal death have also been found in pregnant animals fed with FB_1- contaminated feeds (Voss and Riley, 2013). Studies evaluating the reproductive and developmental toxicity of FB_1 in humans are very rare. However, consumption of FB_1 contaminated feeds have been reported to impair the reproductive potential of Syrian hamsters (Floss et al., 1994), rabbits (Ewuola and Egbunike, 2010), rats (Flynn et al., 1996; Gbore et al., 2012) and chicken embryos (Javed et al., 1993; Bacon et al., 1995; Zacharias et al., 1996). Gbore and colleagues have performed several studies to examine how dietary FB_1 exposure in piglets and boars affect their reproductive function and they observed that such exposure causes a delay in puberty in piglets and impaired sperm production and semen quality of boars (Gbore and Egbunikem, 2008; Gbore et al., 2009a,b).

In humans, fumonisins are reported to be environmental risk factor for birth defects, notably neural tube defects (NTDs). NTDs (spina bifida, exencephaly and craniorhachischisis or meningio-myeloceole) are foetal malformations occurring as a result of failure of the embryonic neural tubes to close properly during the early period of gestation (Voss et al., 2006; Voss and Riley, 2013). Maternal exposure to high

concentrations of FB_1 through the ingestion of contaminated maize and maize-based foods during early pregnancy has been associated with increased risk of NTDs among newborns in regions known to consume maize as staple foods (Melnick and Marazita, 1998; Hendricks, 1999; Marasas et al., 2004; Missmer et al., 2006). In the Transkei region of South Africa and Cameron County, Texas (USA) along the Texas-Mexico border, where maize is a dietary staple and where there is high chronic fumonisin exposure, there have been reports of high incidence of NTDs (Hendricks, 1999; Marasas et al., 2004; Missmer et al., 2006). The incidence of NTDs in these regions is known to be 6-10 times higher compared to the average NTDs incidence rate found globally (~ 10/10,000 live births) (Hendricks, 1999; Marasas et al., 2004; Gelineau-van Waes et a*l*., 2009).

A high incidence of NTDs (27 per 10,000 live births) was observed in the newborns of Mexican-American women living along the Texas-Mexico border who conceived between 1990 and 1991 (Hendricks, 1999). As a result, Missmer et al. (2006) conducted a population-based case-control study among Mexican-American women who delivered newborns affected by NTDs between 1995 and 2000 to evaluate if maternal fumonisin exposure during pregnancy was associated with NTDs in neonates. In this study, Missmer and colleagues assessed serum sphinganine to sphingosine (Sa:So) ratio of 163 Mexican-American women with NTD-affected pregnancies and 189 Mexican-American women who delivered babies without NTDs within the study period. In addition, fumonisin levels were determined from 146 household corn tortillas and 114 tortillas obtained from grocery stores to estimate pre-conceptional dietary fumonisin exposure. The maternal serum Sa:So ratio ranged from 0.08 to 0.36 whereas fumonisin levels in tortillas ranged from non-detectable to 1,690 ng/g. Moderate consumption of tortilla compared to low consumption during the first trimester of pregnancy was associated with increased odd ratio (OR) with NTDs in newborns after adjusting for body mass index, serum B_{12} and folate, and date of conception (OR: 2.4; 95% confidence interval: 1.1 - 5.3). However, higher intakes of tortillas resulted in either a slight decrease in NTDs incidence or absence of NTDs. With regard to the maternal serum Sa:So ratio, higher levels of fumonisin exposure was associated with a higher incidence of NTDs in newborns whereas those with the highest Sa:So ratio (>0.35) had low incidence of NTDs in newborns. It was suggested that at the highest estimated dietary or maternal

serum fumonisin exposure, miscarriages or still birth might have occurred resulting in a low incidence of NTD in this group (Missmer *et al*., 2006). Worthy of note is that this study estimated fumonisin exposure by measuring FB_1 in foodstuffs which has previously failed to provide accurate data on human exposure due to heterogeneous distribution of mycotoxins in food (Shephard *et al*., 2007). In addition, Missmer et al. (2006) applied maternal serum Sa:So ratio as a biomarker of fumonisin exposure. Although serum and urinary Sa:So ratio is suggested as a useful biomarker of fumonisin exposure in animals (Van der Westhuizen et al., 2001), it failed to correlate with human dietary exposure to fumonisins (Van der Westhuizen et al., 2008, 2010). The method of fumonisin exposure assessment applied by Missmer et al. (2006) could have inaccurately estimated fumonisin exposure and results should be interpreted with caution. Therefore, future studies should apply urinary FB_1 biomarker which has been shown to correlate well with human dietary fumonisin exposure (Van der Westhuizen et al., 2011; Shirima et al., 2013; Gong et al., 2015).

In animal studies, FB_1 has been shown to induce NTDs in cultured mouse embryos (Sadler et al., 2002), and altered embryogenesis and caused NTD development in foetuses of pregnant mice exposed during early gestation (Gelineau-van Waes *et al*., 2005). Although FB_1 is suggested as an etiologic agent in the development of NTDs, other factors including genetics and maternal nutrition play an important role in pregnancy outcome and dietary folic acid supplementation prior and during pregnancy is known to reduce the occurrence of NTDs (Greene and Copp, 2005). The potential for FB_1 to cause NTDs is primarily attributed to its structural similarity with sphingamine and sphingosine (Gelineau-van Waes et al., 2005). FB_1 and other fumonisins competitively interfere with ceramide synthase which is involved in the *de novo* synthesis of complex glyco-sphingolipids resulting to the accumulation of sphingamine and sphingosine in tissues, serum and urine (Merrill et al., 2001; Bolger et al., 2001; Riley and Voss, 2006; Voss et al., 2005, 2006). It has been postulated that NTDs are induced by fumonisins as a result of decreased concentration of complex sphingolipids, disruption of lipid rafts and impairment in the function of the high affinity placental carriers [folate binding protein 1 (folbp1) in mice and folate receptor alpha (FRα) in humans] involved in folate transport causing folate deficiency in the foetus during early pregnancy and failure of the foetal neural tube to close properly (Voss et al., 2001;

Gelineau-van Waes et al., 2005, 2009, 2012; Voss and Riley, 2013). In addition, the complex glycol-sphingolipids are important in the maintenance of cell membrane integrity, cell growth and migration, cell differentiation, cell morphology and endothelial cell permeability and therefore, disruption of sphingolipid metabolism by fumonisins can negatively affect embryonic morphogenesis and cell apoptosis (Merrill et al., 2001; Yahia and Kamata, 2017). Based on these studies discussed above, the negative impacts of FB_1 on reproductive function in animal and *in vitro* models cannot be ignored as there is a homology in organ systems between animals and humans.

1.2.4 Trichothecenes

The trichothecenes are toxic sesquiterpenoid mycotoxins produced by *Fusarium*, *Stachybotrys* and *Myrothecium species* of fungi during their growth in food and/or the environment (Pestka, 2010a,b). The broad family of trichothecenes have been classified into four groups Types A, B, and D based on the substitution pattern of the tricyclic 12, 13 epoxytrichothec-9-ene core structure (Escrivá et al., 2015). Type A trichothecenes include T-2 toxin, HT-2 toxin, neosolaniol and diacetoxyscirpenol, whereas the type B group are fusarenon-X, nivalenol (NIV) and deoxynivalenol (DON) and its 3-acetyl and 15-acetyl derivatives (**Fig. 1.4**). The detailed toxicological effects and molecular mechanisms of trichothecenes toxicity has been reviewed in Pestka, (2010a,b) and Escrivá et al. (2015).

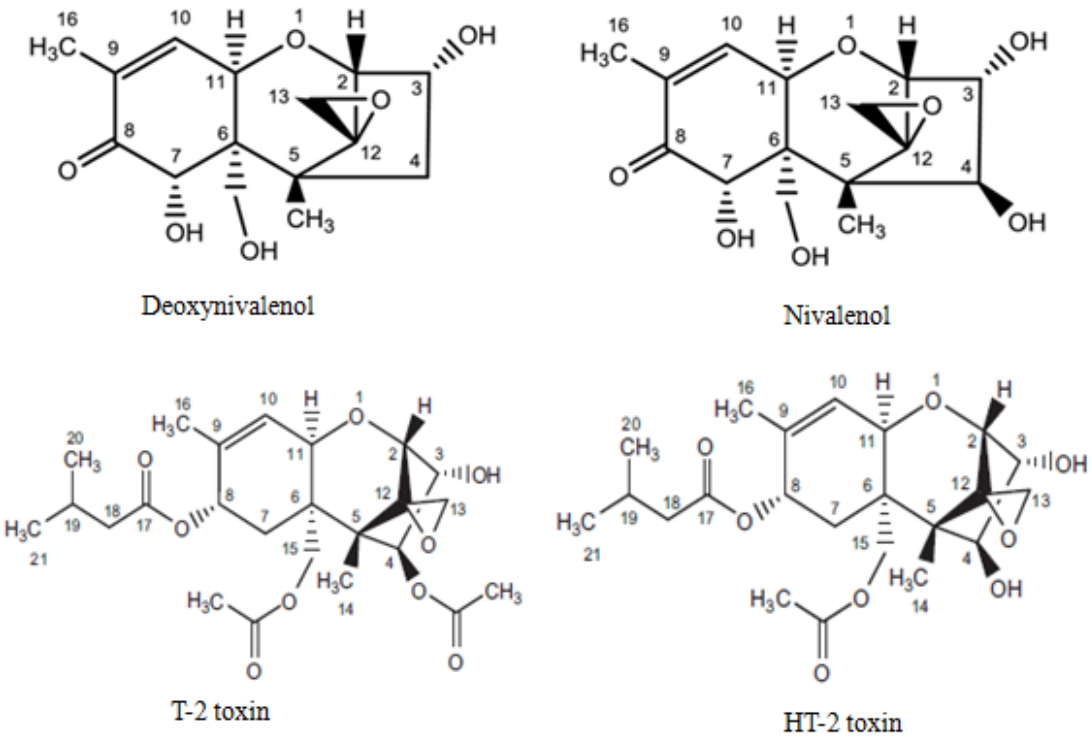

Fig. 1.4: Chemical structures of major trichothecenes (Source: EFSA, 2017c,d)

1.2.4.1 Deoxynivalenol

Deoxynivalenol (DON; **Fig. 1.4**) is one of the type B trichothecenes and it is known to cause disruption of gastrointestinal permeability, impair growth hormone signalling, dysregulation of the expression of genes involved in inflammatory response, immunotoxicity, haematological disorders, and alteration of the neuroendocrine responses in humans and animals (Pestka, 2010a,b). The molecular mechanisms underlying these pathological effects involve the causation of oxidative damage, induction of apoptosis and autophagy, alteration of membrane integrity, and inhibition of DNA, RNA and protein synthesis (Pestka, 2010b; Payros et al., 2016). The effects of DON on human reproduction and development are scarce, however, there are reports on its adverse effects on reproduction and development in animal and *in vitro* models (Yu et al., 2017a).

DON has been implicated in the disruption of oocyte maturation, embryo resorption and foetal malformation in mice (Khera et al., 1982, 1984; Hou et al., 2014; Yu et al., 2017b), rats (Collins et al., 2006), and swine (Alm et al., 2002, 2006). In a study to

assess the toxic effects of DON on mouse embryo, Khera *et al*. (1982) exposed pregnant mice in their gestation days (GD: 8 – 11) to DON (0.5 – 25 mg/kg/body weight (b.w)) through oral gavage. DON at 0.5 mg/kg/b.w had no maternal and foetal toxicity, however, at 1, 2.5 and 5 mg/kg/b.w DON caused foetal malformation. In addition, there were embryo resorptions in mice treated with 5, 10 and 25 mg/kg/b.w whereas significant reduction in live foetuses were observed at >5 mg/kg/b.w. In a subsequent study, maternal toxicity, foetal malformation and embryo resorption were reported in mice and rats exposed to DON at >1 mg/kg/b.w (Khera et al., 1984; Debouck et al., 2001). Similarly pregnant rabbits fed with DON contaminated diet showed 100% incidence of embryo resorption in the 1.8- and 2.0-mg/kg groups and decreased mean foetal weight in the 1.0- and 1.6-mg/kg group (Khera et al., 1986). However, none of the doses caused foetal malformation or showed teratogenic potential. In accordance with the above results, Hou and colleagues found that exposure of mice with DON-contaminated maize (3.875 mg/kg/diet) for 4 weeks resulted in poor oocyte indices and low developmental competence of the ovaries (Hou et al., 2014). Taken together, these studies indicate that a high exposure to DON during pregnancy could directly induce foetal malformation or indirectly affect embryonic and foetal development as a result of maternal toxicity (Collins et al., 2006). With regard to studies on rats treated orally with DON, the CONTAM Panel (EFSA, 2017a) set the no-observed-adverse-effect-level (NOAEL) as 1.0 mg/kg b.w per day for reproductive endpoints, 1.0 mg/kg bw per day for foetal toxicity and 0.5 mg/kg bw per day for maternal toxicity.

The toxicity of DON to male germ cells in animals have been reported. For instance, exposure of IL-6KO and B6C3F1 mice strains to DON (2.5 – 5.0 mg/kg) induced testicular germ cell degeneration, decreased absolute caudal epididymal sperm numbers, a reduction in caudal epididymal weights, and reduced serum testosterone levels (Sprando et al., 1999, 2005). This is supported by an *in vitro* study in which exposure in MA-10 murine Leydig cell line to DON (0.25 – 5 µM) increased the release of reactive oxygen species, significantly reduced cell viability and caused significant decline in forskolin-induced progesterone secretion after 24 hours of exposure (Savard et al., 2016).

Urinary concentrations of 436 ng/mL (1.471 µM) and 1,238 ng/mL (4.178 µM) have been reported for DON and its conjugates in pregnant women (Sarkanj et al., 2013; Wells et al., 2016) and therefore, these concentrations have clinical relevance. DON can be transferred through the placenta to the foetus in pregnant sows (Goyart et al., 2007; Dänicke et al., 2007) and it is likely that human foetus will be exposed to DON during pregnancy. In an *in vitro* placental perfusion using BeWo cell line, Nielsen et al. (2011) showed that DON was transported across the membrane and caused a dose-dependent decrease in the secretion of beta-human chorionic gonadotrophin (β-hCG). Normal β-hCG levels are required for the proper functioning of the placenta and foetal development, therefore the reduction in this important hormone further supports the embryo resorption, foetal death and malformation reported in animal studies. In addition, it has been argued that DON exposure in females causes the accumulation of reactive oxygen species, induction of apoptosis, and alteration of epigenetic modification in the female reproductive system resulting to DON-induced reproductive and developmental toxicity (Han et al., 2016; Yu et al., 2017b). Apoptosis (also known as programmed cell death) is a process through which the body controls normal development and tissue homeostasis in adult tissues (Ma et al., 2012). It is characterised by morphological changes in cells such as nuclear shrinkage and fragmentation, chromatin aggregation, fewer and even absence of microvilli, mitochondrial swelling, presence of apoptotic bodies and vacuolisation (Ma et al., 2012). Several mediators are involved in apoptosis, including cysteinyl aspartate-specific proteases (caspases), b-cell lymphoma 2 protein, b-cell lymphoma-2-associated x protein, poly (ADP-ribose) polymerase, apoptosis inducing factor mitochondria associated 1, p53 tumour suppressor protein, apoptosis protease activating factor 1, and cytochrome c (Khan and Mukhtar, 2010). For apoptosis to be induced, the bax protein forms a heterodimer with bcl-2 protein leading to the loss of mitochondrial membrane potential and the release of cytochrome c to the cytosol. Cytosolic cytochrome c acting in conjunction with apoptosis protease activating factor 1 activates caspase-9 which stimulates subsequent caspase-3 or caspase-7 resulting in apoptosis (Khan and Mukhtar, 2010). In addition, apoptosis can also indirectly be induced by p53 gene since the expression of bax is regulated by p53. In human colon cancer cells (HT-29) exposed to DON at 125 – 2000 ng/mL, it was reported that cell apoptosis induced by DON involves the activation of caspases (especially caspase-9, caspase-8 and caspase-3) through the inhibition of bcl-2 expression and modulation of bax protein expression, which lead to the release of

cytochrome c from the mitochondria into the cytosol (Ma et al., 2012). In a different study, DON exposure caused DNA damage in HT-29 cells and increased p53 protein level resulting in the induction of caspase 3 activity initiating apoptosis (Bensassi et al., 2009). DON distorted mitochondrial membrane potential and also induced the expression of apoptosis related genes such as caspase-3, caspase-8, caspase-9, and apoptosis inducing factor mitochondria associated 1 in DF-1 chicken embryo fibroblasts (Li et al., 2014d). In a more recent study, DON (100 – 1600 ng/mL) exposure also caused an increase in the expression of caspase-9, caspase-3 and poly (ADP-ribose) polymerase and the bax/bcl-2 ratio in mouse endometrial stromal cells in vitro (Dai et al., 2017). Taken together, it can be deduced that DON causes mitochondrial-mediated cell apoptosis through the induction of DNA damage which leads to the induction of p53 tumour suppressor gene and activation of caspases.

It has also been speculated that DON exposure could cause epigenetic modification in oocytes, which leads to the alteration in oocyte quality and developmental competence. In porcine oocytes treated with 3 µM of DON *in vitro*, it was observed that increased global DNA methylation, expression of *DNMT3A* mRNA, H3K27me3 and H3K4me2 protein levels were observed in cells exhibiting poor quality and reduced developmental competence (Han et al., 2016). The authors concluded that epigenetic modification might be the possible mechanism through which DON inhibited the developmental competence of the porcine oocytes (Han et al., 2016). However, further studies is required better understand this proposed mechanism of DON toxicity in oocytes.

In summary, it is plausible that human exposure to DON either *in utero* or during post-natal period can impair reproduction and development. Therefore, properly designed *in vitro* and *in vivo* bioassays as well as human epidemiological study is required to provide stronger evidence establishing the association between exposure to DON and adverse reproductive health outcomes in humans.

1.2.4.2 Nivalenol

Similar to DON, the effects of nivalenol (NIV; **Fig. 1.4**) on reproduction and development have also been reported (Pestka, 2010a), although there is no literature on

the reproductive health effects in human beings. Ito and colleagues reported that intraperitoneal administration of NIV (0.1 – 1.5 mg/kg bw/day) to pregnant ICR mice from 7 – 15 days of gestation caused vaginal bleeding in 60% of the exposed mice, stillbirths and induced 48 – 88% embryo deaths, especially at 0.5 and 1.5 mg/kg bw (Ito et al., 1986). It was also observed that the injection of 3 mg NIV/kg b.w on gestation day 7 was highly toxic to both the embryo and placenta culminating in stillbirths within 48 hours. In a similar study, maternal and embryo toxicity which resulted in intrauterine growth retardation was reported when pregnant mice (GD: 7 – 15) were either fed with 6 – 30 mg/kg/diet of NIV or 1 – 20 mg/kg b.w of NIV by oral gavages, particularly at the highest doses (Ito et al., 1988). Teratogenic effects were not observed in any of the concentrations of NIV used either in contaminated feeds or oral gavage (Ito et al., 1988). When female F344/DuCrj rats were fed diets containing NIV at 6.25, 25 and 100 mg/kg for 90 days, histopathological features showed an increase in atretic ovarian follicles and interstitial glands, impaired corpora lutea development, and uterine atrophy with diestrus endometrial mucosa change in those fed the highest dose (100 mg/kg) (Takahashi et al., 2008; Sugita-Konishi et al., 2008). However, there was no adverse effect on the primary and secondary follicles. On the other hand, there was no effect on the reproductive system in male F344/DuCrj rats fed with same diet containing the same amount of NIV (Takahashi et al., 2008; Sugita-Konishi et al., 2008). In contradiction to the above studies, pregnant sows fed with maize contaminated with NIV did not affect any reproductive parameters assessed (Williams and Blaney, 1994). It can be deduced that female rodents are more susceptible to NIV than their male counterparts and pigs.

1.2.4.3 T-2 and HT-2 Toxins

Similar to DON, T-2 and its metabolite HT-2 (**Fig. 1.4)** were shown to cross the placenta in a BeWo cell placental perfusion model and impair BeWo cell proliferation, indicating their ability to cause placental, embryonic and foetal toxicity (Wang et al., 2013). T-2 was also found to inhibit porcine granulosa cell proliferation and significantly inhibited progesterone (P4) and oestradiol (E2) in the presence of follicle stimulating hormone (FSH) and IGF1 stimulation *in vitro* (Caloni et al., 2009). This is in disagreement with an *in vitro* study which showed that T-2 and HT-2 toxins in the presence of IGFI upregulated P4 release by porcine ovarian granulosa cells and this

potentially affect steroidogenesis in the ovaries (Maruniakova et al., 2014) and in H295R cell line where lower doses reduced hormone levels whereas higher doses induced hormone (progesterone, oestradiol, testosterone and cortisol) levels (Ndossi et al., 2012). The differences could be as a result of culture media composition (e.g. serum, amino acids, antibiotics, etc) and duration of incubation as these are well established to affect the hormone production *in vitro* studies. For instance, the Nu-serum in the growth media of H295R cells have been shown to contribute substantial amount of progesterone and testosterone which may give false increase in progesterone and testosterone levels in the assay (Zhang et al., 2011; Nakano et al., 2016; Strajhar et al., 2017).

Epigenetic modification of oocytes and induction of apoptosis could be another critical molecular mechanism through which T-2 and HT-2 toxin induce poor oocyte maturation and embryo toxicity (Wu et al., 2013, 2015; Zhang et al., 2016, 2017). For instance, the exposure of porcine oocytes to 100 nM of HT-2 for 48 hours in maturation medium reduced embryo developmental competence by modifying both DNA and histone methylation (Zhang et al., 2017), and caused oxidative stress induced apoptosis and autophagy leading to the disruption of actin distribution and meiotic spindle morphology of developing oocytes (Zhang et al., 2016). In a subsequent study, embryos from six-week-old BDF1 female mice exposed to 0.5, 0.75, and 1 ng/ml T-2 were cultured *in vitro* for 96 h and there was decrease in blastocyst proportion, impeded blastulation, and increased the rate of chromatin damage, especially those from mice exposed to 0.75 and 1 ng/ml T-2 toxin (Somoskői et al., 2016a,b). When male mice were given 0.78 - 0.99 mg/kg body weight T-2 toxin by gavage for 3 days, increased abnormal sperm morphology, reduction in sperm motility, decreased in seminal plasma citric acid level, and decline in testosterone concentrations were observed (Kovács et al., 2011). Moreover, T-2 toxin significantly impaired testosterone production in gerbil testicular interstitial cells (Fenske and Fink-Gremmels, 1990) and caused poor sperm quality in mice (Yang et al., 2010). Based on the results discussed above, it can deduced that T-2 and its metabolite HT-2 could cause reproductive and developmental toxicity through the dysregulation of steroidogenesis, induction of apoptosis and autophagy and epigenetic modification. Therefore, future human research should consider T-2 - and

HT-2-induced autophagy and epigenetic alteration, which indirectly influence the reproductive system and foetal development.

1.2.5 Zearalenone

Zearalenone (ZEN) produced by *Fusarium species* is also considered as one of the common mycotoxins posing a threat to human and animal reproductive health (Zinedine et al., 2007). ZEN is primarily converted by hydroxysteroid hydrogenases to phase I metabolites, including α-zearalenol (α-ZOL), β-zearalenol (β-ZOL), α-zearalanol (α-ZAL), β-zearalanol (β-ZAL) and zearalanone (ZAN) (**Fig. 1.5**). ZEN and the metabolites are known endocrine disruptors due to their resemblance to 17β-oestradiol, enabling them to disrupt the binding of 17β-oestradiol to oestrogen receptors. Both *in vivo* and *in vitro* studies show that α-ZOL has higher oestrogenic potency compared to ZEN, whereas β-ZOL has lower oestrogenic potency (EFSA, 2017b). Both ZEN and α-ZOL have been reported to cause hyper-oestrogenism with attendant reproductive disorders in farm animals (Zinedine et al., 2007). In animals, ZEN is known to reduce testosterone levels and induce abnormal sperm quality in males (Yang et al., 2007; Zatecka et al., 2014), and cause ovarian follicle atresia, uterine hyperplasia, and degeneration of oocyte meiotic chromatin in females (Yamini et al., 1997; Obremski et al., 2003; Skorska-Wyszyńska et al., 2005; Alm et al., 2006). Despite the evidence that ZEN causes adverse effects on reproduction in animals, there are currently no published reports of associations of ZEN with infertility or birth defects in human populations.

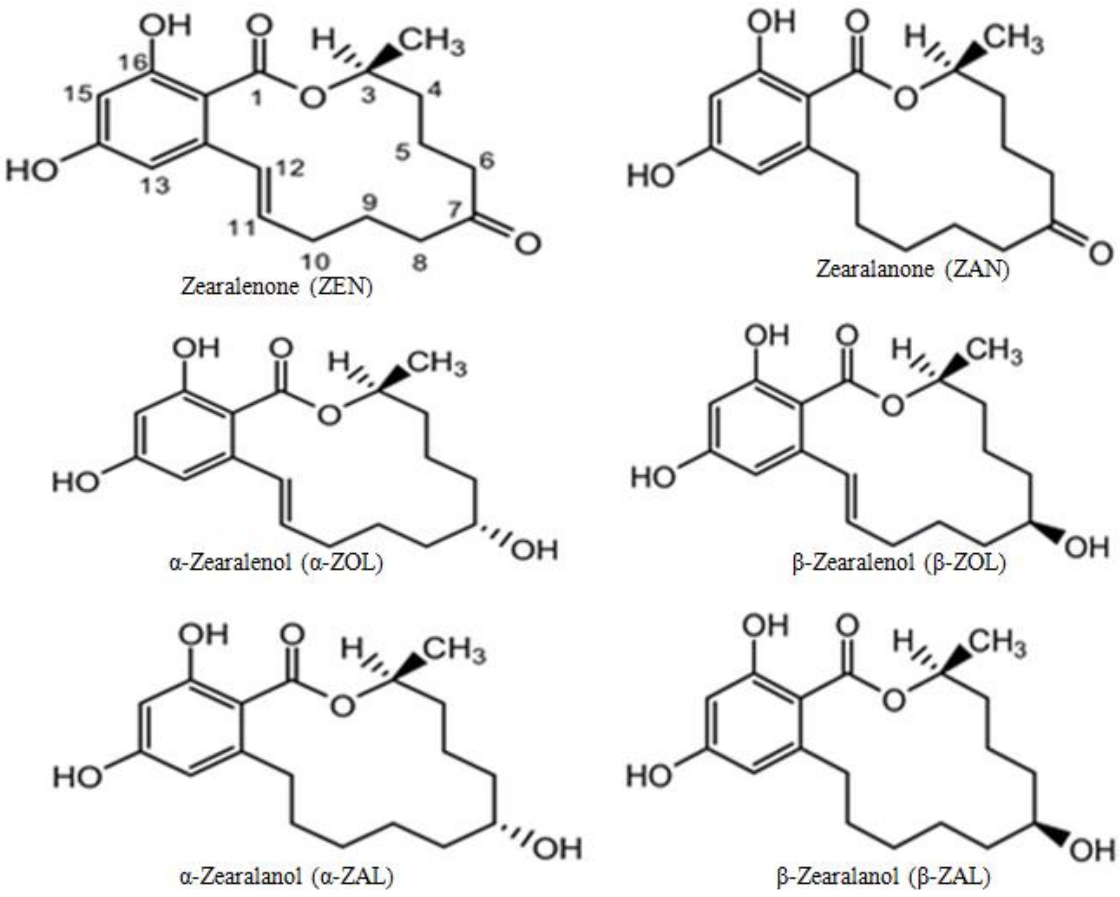

Fig. 1.5: Chemical structures of zearalenone and its major metabolites (Source: EFSA, 2016)

There have been some reports of the association of ZEN exposure with central precocious puberty (CPP), premature thelarche, and hyperplastic and neoplastic endometrium (**Table 1.1**). In a study comprising 32 girls with CPP and 31 healthy female controls, elevated levels of ZEN and α-ZOL were detected in the serum of six out of the 32 cases (Massart et al., 2008, 2010). In response to a high incidence of CPP in the Viareggio region of North-West Tuscany in Italy, Massart et al. (2008) recruited 32 girls (17 from Viareggio region and 15 from Pisa) with idiopathic CPP visiting the Paediatric Endocrine Centre of Pisa and matched them with 31 healthy female controls. In these groups, ZEN and its metabolites were assessed and matched with height, weight and height velocity, body mass index, bone age, and gonadal secretion. Among the 17 CPP patients from Viareggio region, 6 (35%) had detectable levels of ZEN (mean: 933.7 ± 200.3 pg/mL) and α-ZOL (mean: 106 ± 1.9 pg/mL) whereas none of the 15 CPP patients from Pisa had ZEN or its metabolites detected in their serum. It was also observed that the six girls with ZEN and α-ZOL exposure had higher height, weight

and height velocity after 12-month triptorelin (gonadotrophin-releasing hormone agonist) treatment compared to the 26 CPP patients and 31 healthy controls with non-detectable serum ZEN and α-ZOL (Massart et al., 2008). As a result of these parameters, ZEN and α-ZOL exposure was suggested as predisposing factor of idiopathic CPP in prepubertal girls. However, the ZEN and its metabolites could not be detected in the other 26 girls with CPP giving an inconclusive result and therefore, other environmental contaminants may have been predisposing factors of the reported CPP (Massart et al., 2008, 2010). Previously, ZEN exposure was also speculated to be associated with early breast development among young Hungarian girls (Szuets et al., 1997). In this report, five of 36 early telarche/mastopathy patients in the South-East Region of Hungary had high serum level of ZEN (18.9 - 103.5 µg/mL) and it was speculated that ZEN could be a contributory factor for the development of early telarche/mastopathy (Szuets et al., 1997). However, this study could not clearly state statistically how the concentration of ZEN was related to early telarche/mastopathy development and so, should be interpreted with caution.

Other studies also suggest that ZEN may be a contributory factor to premature thelarche and idiopathic CPP in prepubertal girls (Schoental, 1983; Deng et al., 2012; Asci et al., 2014). In contradiction to these studies above, Bandera and colleagues conducted a cross-sectional study comprising 163 girls (age: 9 - 10 years) from New Jersey in USA to evaluate the effects of exposure to ZEN and its metabolites on body size and breast development (Bandera et al., 2011). In this study, 78.5% of the girls had urinary ZEN (0.05 - 33.12 ng/mL), α-ZOL (0.003 - 10.69 ng/mL), β-ZOL (0.05 - 1.10 ng/mL), α-ZAL (0.02 - 0.57 ng/mL), β-ZAL (0.04 - 0.60 ng/mL) and ZAN (0.07 - 3.31 ng/mL), with those who consumed beef and popcorn the day prior to urine collection having significantly higher concentrations compared to those who did not take these foods. Girls who had detectable levels of urinary ZEN and its metabolites had lower height and delayed onset of breast development after adjusting for age, body mass index, isoflavone intake, and recruitment year, and it was concluded that exposure to ZEN and its metabolites had anti-oestrogenic effects on the girls (Bandera et al., 2011). The difference in the results reported in this study compared to the previous studies could be attributed to the small sample size of ZEN exposure (5 or 6), detection of ZEN and metabolites in serum instead of urine, and having subjects comprising of girls undergoing CPP treatment (Bandera et al., 2011). Therefore, well designed longitudinal

studies with larger sample size, the inclusion of people with different ethnic background and countries, and the use of valid ZEN exposure biomarkers would certainly provide stronger evidence for establishing a causal association or not. In addition, reproductive hormones such as oestrogen, progesterone, follicle stimulating hormone, and luteinising hormone should be evaluated and included in the statistical analysis as they can also influence the onset of puberty in girls.

However, biologically relevant doses of ZEN (5 - 10 mg/kg bw) caused precocious puberty in immature female rats as a result of early induction of the hypothalamic kisspeptin (KISS1)-G-protein coupled receptor-54 (GPR54) signalling pathway which is responsible for the initiation of puberty onset, regulation of the hypothalamo-pituitary-gonad (HPG) axis and reproductive function (Kriszt et al., 2015; Yang et al., 2016). The precocious puberty in treated rats were evidenced through the induction of *Kiss1*, *GPR54* and *GnRH* (Gonadotropin-releasing hormone) expressions in the hypothalamus at both mRNA and protein levels (Yang et al., 2016). This could be the same mechanism through which ZEN causes precocious puberty in human beings since mutations and/or inactivation of the *GPR54* and/or *KISS1* genes in humans and mice (*gpr54* and *kiss1*) has been linked to reproductive dysfunction, including delayed puberty onset, abnormal oestrus cycles and infertility (Semple et al., 2005; Kirilov et al., 2013).

1.2.6 Ochratoxin A

Ochratoxins are mainly produced by *Aspergillus* and *Penicillium* species and contaminate cereals, coffee, dried fruit and other products. Major ochratoxins include Ochratoxin A and Ochratoxin α (**Fig. 1.6**). Other forms are its methyl ester, its ethyl ester (Ochratoxin C), 4-hydroxyochratoxin A, Ochratoxin B with its methyl and ethyl esters. Ochratoxin A (OTA) is the most toxic of all the ochratoxins and studies shows that OTA is nephrotoxic (associated with Balkan Endemic Nephropathy), hepatotoxic, neurotoxic, immunotoxic, genotoxic and carcinogenic, and have been classified as a possible human carcinogen (group 2B; IARC, 2002).

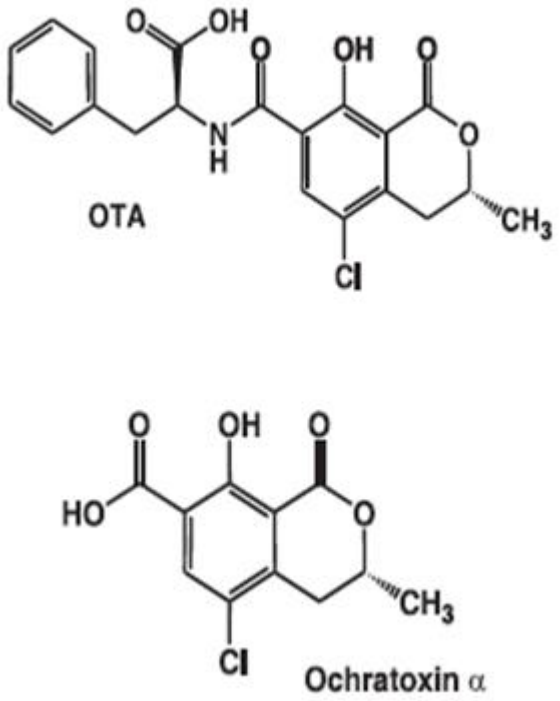

Fig. 1.6: Chemical structure of ochratoxin A (OTA) and ochratoxin α (Source: Mally, 2012; Reproduced with permission obtained from the publisher Oxford University Press)

Several studies have examined the possible effect of ochratoxin A (OTA) on reproduction using either *in vivo* or *in vitro* models (reviewed by Malir et al., 2013), but only one has related its impact on human fertility through *in vitro* exposure of normal human spermatozoa to OTA (Chakraborty and Verma, 2009). In five male Wistar rats treated with OTA 289 µg/kg b.w by gastric intubation for 8 weeks, testicular α-amylase, alkaline phosphatase and γ-glutamyltransferase (γGT) were all decreased (Gharbi et al., 1993). A 2 fold increase of the testosterone level in testes was observed in the OTA treated rats. The decrease in the testicular enzymes suggests an impairment of spermatogenesis and an accumulation of pre-meiotic germinal cells may be induced by OTA. In another study, the oral administration of albino Swiss mice with OTA (1 µg/kg b.w/day) for 45 days increased the production of abnormalities in both mitotic and meiotic chromosomes, and the gross morphology of the sperm head (Bose and Sinha, 1994). Verma and Chakraborty, (2008) administered 50 and 100 µg of OTA (in 0.2 mL olive oil/animal/day) orally to adult male albino mice for 45 days. Lipid

peroxidation was found significantly increased whilst GSH (glutathione), TAA (total ascorbic acid), SOD (superoxide dismutase), CAT (catalase), GPX (glutathione peroxidase), GRX (glutathione reductase) and GST (glutathione transferase) decreased in the testis when compared to controls (Verma and Chakraborty, 2008). It has been reported that testicular CAT is a very useful Sertoli cell marker and testicular CAT activity is well correlated with relative numbers of Sertoli cells (Verma and Chakraborty, 2008). Therefore, a decline in CAT activity will cause a drastic reduction in the numbers of Sertoli cells thereby affecting spermatogenesis directly or indirectly. So a decline in CAT activity in the testis may lead to infertility in ochratoxin-treated animals and possibly human beings chronically exposed to OTA contaminated food. In a recent study, Chakraborty and Verma (2009) orally administered ochratoxin (50 and 100 μg/0.2 mL of olive oil/animal/day) to male albino rats for 45 days. After 45 days treatment, it was observed that there was a significant decline ($p < 0.05$) in cauda epididymal sperm count, sperm motility, sperm viability and fertility rate which were dose-dependent. In addition, they treated normal human sperm cell suspension with OTA and reported significant alterations in sperm motility, sperm viability and sperm morphology (swollen head, swollen head and mid-piece, coiled tail, decapitation, head-tail agglutination and head-head agglutination) which were both time-dependent and concentration-dependent.

Structural and numerical aberrations of spermatocytes in OTA-treated albino mice (1.8 mg/kg b.w.) have been reported (Farag et al., 2010). A significant reduction in the mitotic and meiotic activities of somatic and germ cells were observed. Moreover, OTA caused a significant decline in cauda epididymal sperm count, sperm motility and increased sperm abnormalities compared to control (Farag et al., 2010). In a different study, Kumar et al., (2011) administered OTA contaminated feed (4 mg/kg) orally to mature male wistar rats for 30 days and reported a significant decrease in their testosterone levels. Hassan and colleagues investigated the influence of AFB_1, ZEN and OTA on hormones associated with fertility in male albino rats and feed contaminated with AFB_1 (0.5 ppm), OTA (1.0 ppm) and ZEN (2.5 ppm) were given to male albino rats for up to 6 months (Hassan et al., 2010). After 5 months of treatment, OTA, ZEN and AFB_1 significantly reduced the serum levels of leutinising hormone (LH), follicle stimulating hormone (FSH) and testosterone. Interestingly, Fenske and Fink-Gremmels

(1990) investigated the direct effect of mycotoxins, including OTA on testicular function by treating dispersed interstitial cells from testes of adult gerbils cultured with different concentrations of OTA and reported that high concentrations of OTA (1838 μM) inhibited steroidogenesis and testosterone production, but this report should be interpreted with utmost care as human beings are unlikely to be exposed to such high concentration of OTA. In a subsequent study, Biro et al. (2003) investigated the effect of OTA on the sperm quality of boars. The animals were given 20 mg OTA orally daily for 6 weeks, followed by a 9-week withdrawal period. They measured spermatozoa motility at 0, 24, 48, 72 and 96 h after the withdrawal period, and ejaculation volume, initial viability and progressive motility were recorded. The study demonstrated that OTA may affect sperm production and boar semen quality but this was seen only after a lag period. OTA was found in the seminal plasma of the boars; however, OTA treatment did not affect the morphology and number of Leydig cells nor the epididymal structure. The results suggest that OTA may negatively influence sperm production and boar semen quality (Biro et al., 2003). In accordance with previous studies, Solti et al. (1999) reported that boars fed with OTA at 5 and 10 times of the human tolerable daily intake (equivalent to 16 ng OTA/kg b.w per day) have high concentration of OTA in both their seminal plasma and serum at day 35 after toxin challenge than the control group, and OTA profiles correlated to serum profiles of the OTA treated boars. There was also a sharp reduction in the sperm motility in the treated animals compared to controls suggesting that OTA may negatively affect spermatozoa quality (Solti et al., 1999). The poor semen parameters of animals exposed to OTA could be possibly due to the structural similarity of OTA with phenylalanine (Phe) and is therefore, a competitive inhibitor of Phe-dependent enzymes including phenylalanyl-tRNA synthetase (Creppy et al., 1979a). Phenylalanyl-tRNA synthetase is one of the aminoacyl synthetases responsible for the transfer of amino acids to their respective transfer RNA giving rise to the generation of the genetic code which is very important in protein synthesis and cell viability (Creppy et al., 1979b). Therefore, the inhibition of phenylalanyl-tRNA synthetase by OTA impairs protein synthesis (Creppy et al., 1979a). It is expected that such interference in the protein synthesis could affect spermatogenesis, maturation of spermatozoa and causes abnormal stability of spermatozoa membrane resulting to poor spermatozoa quality.

Some studies on the endocrine disrupting effects of OTA have also been conducted using *in vitro* cell lines. Frizzell et al. (2013) treated human adrenocortical carcinoma cells (H295R cells) with different concentrations of OTA (0.1-1000 ng/ml) and reported that OTA at 1000 ng/ml caused a 3-fold increase in oestradiol production (117 ± 14 ng/ml) compared to the control (36 ± 9pg/ml). In another study, OTA has been shown to cause increase in progesterone production and also upregulates the activity of 3β-hydroxylsteroid dehydrogenase (3β-HSD) in human choriocarcinoma cell line (JEG-3 cells) by 281–378% at 72 and 96 h (Woo et al., 2013). Taken together, the reports show that OTA has the potential of causing reproductive and developmental toxicity.

1.2.7 Mycotoxin co-exposure and implications for human reproductive health

Different concentrations of mycotoxin biomarkers have been reported globally in human exposure studies **(Table 1.2 - Table 1.5)**. Recent advances in liquid chromatography tandem mass spectrometry (LC-MS/MS) have revolutionised mycotoxin biomarker assessment through the analysis of multiple mycotoxins in one sample injection. As a result, multiple mycotoxin exposure biomarkers have been reported in several population studies which show that human beings are often simultaneously exposed to mixtures of mycotoxins (**Table 1.6**). For instance, in the former Transkei region of South Africa, Shephard and colleagues examined 53 urine samples and demonstrated that mixtures of biomarkers such as deoxynivalenol (DON), zearalenone (ZEN), fumonisin B_1 (FB_1) and ochratoxin A (OTA) as well as their metabolites can be detected frequently in the human urine samples (Shephard et al., 2013). Similarly, out of 220 urine samples obtained from children in Cameroon, 160 (73%) had biomarkers for OTA, DON, AFM_1, FB_1, ZEN, α-ZOL and β-ZOL. Co-occurrence of 2, 3 and 4 mycotoxins was 35%, 5% and 5%, respectively (Ediage *et al*., 2013). In a recent study in Nigeria, mycotoxins and their metabolites, including AFM_1, DON, OTA, FB_1, FB_2, FB_3, ZEN, zearalenone-14-O-glucuronide (ZEN-14-GlcA) and deoxyvalenol-15-O-glucuronide (DON-15-GlcA) were detected in 51% of urine samples from 120 volunteers involving children, adolescents and adults, and 25% of them indicated multi-mycotoxin exposure (Ezekiel et al., 2014). In another study in Italy (Solfrizzo et al., 2014), biomarkers of ZEN + ZOLs (100%), OTA (100%), DON (96%), FB1 (56%), and AFM_1 were found in urine samples with 52% exposures involving mixtures of DON, ZEN, FB_1 and OTA. Furthermore, in a three-year survey of

the global occurrence of mycotoxins using 7049 feed and feedstuff samples, it was reported that 48% were contaminated by two or more mycotoxins (Rodrigues and Naehrer, 2012). Globally, the rate of co-occurrence of AFB_1, DON, ZEN, OTA and fumonisins in feeds and feedstuff continues to be high (Schatzmayer and Streit, 2013) and therefore, requires toxicological consideration. Despite the fact that human beings can be exposed to a range of mixtures of mycotoxins, most toxicological studies have only taken into account of the effects of exposure to a single mycotoxin. However, combinations of mycotoxins can be agonistic, additive or antagonistic, and therefore, could pose a significant threat to human reproductive health (Alassane-Kpembi et al., 2013).

Epidemiological research evaluating the effects of mycotoxin mixtures on human reproduction is currently scarce, however, there are few *in vitro* studies evaluating their effects on the female and male reproductive system. Pizzo and colleagues examined the negative effects of single and combinations of DON, α-zearalenol (α-ZOL) and β-zearalenone (β-ZOL) on cell proliferation, steroidogenesis and gene expression of bovine small-follicle granulosa cells after 48 hours exposure *in vitro* (Pizzo et al., 2016). The presence of α-ZOL (3.1 μM) and β-ZOL at 31 μM significantly inhibited the growth of the bovine small-follicle granulosa cells whereas DON (0.1 – 3.3 μM) had no significant effects on the graulosa cell proliferation. Worthy of note is that the exposure of the granulosa cells to binary combinations of α-Zol (3.1 μM) and DON (3.3 μM) significantly increased cell proliferation, indicating synergistic effects. In addition, combination of α-ZOL with DON or β-ZOL inhibited oestradiol secretion higher than the effects obtained in single treatment of each of the toxins, but had no significant effect on progesterone production. In the same study (Pizzo et al., 2016), co-exposure of DON and β-ZOL to the granulosa cells decreased cell proliferation, and strongly inhibited oestradiol production. Similarly, DON with α-ZOL or β-ZOL caused a significant up-regulation in the expression of *CYP11A1* in IGF1 stimulated granuolsa cells. In a different study by Pizzo and colleagues (Pizzo et al., 2015), the individual and combined effects of DON and α-ZOL on cell proliferation and steroidogenesis of FSH plus IGF1 stimulated bovine large follicle granulosa cells was examined after 48 hours of exposure. It was observed that DON (3.3 μM) significantly reduced cell proliferation by 22% whereas α-ZOL (0.09 and 0.31 μM) stimulated large granulosa cell growth. Interestingly, binary combination of DON (0.01 μM) and α-ZOL (0.09 μM) stimulated

cell proliferation by 45% when compared to vehicle control, although this was not significantly different from the stimulatory effect of single treatment with α-ZOL (0.09 μM). It was also reported that that DON (0.33 and 3.3 μM) significantly impaired both oestradiol and progesterone production compared to controls. Interestingly, co-exposure of DON and α-ZOL to the granulosa cells in the presence of FSH inhibited progesterone secretion, but induced oestradiol production in the granulosa cells (Pizzo et al., 2015). Taken together, it can be deduced that co-exposure of DON with α-ZOL or β-ZOL at concentrations relevant to human exposure can impair bovine ovarian cell proliferation and steroidogenesis which may negatively affect normal follicle development and oocyte function. Therefore, human research should be taken to elucidate the molecular mechanisms through which co-exposure of DON with α-ZOL or β-ZOL affect ovarian function and morphology.

In a subsequent study, Albonico et al. (2016) evaluated the effects FB_1 in single and combined with DON, α-ZOL or β-ZOL on bovine granulosa cells in the presence of IGF1 *in vitro*. FB_1 (30 ng/mL – 5 μg/mL), α-ZOL (5 μg/mL) and β-ZOL (30 ng/mL) had no significant effect on bovine granulosa cell growth. However, a combination of FB_1 (30 ng/mL) with same concentration of β-ZOL significantly stimulated cell growth. At higher concentrations, FB_1 (5 μg/mL) strongly reduced cell numbers, and co-exposure of FB_1 (5 μg/mL) with β-ZOL (5 μg/mL) also inhibited cell numbers. It was also reported that co-treatment of FB_1 with α-ZOL caused a significant decline in granulosa cell numbers, indicating either additive or synergistic effects as none of the mycotoxins had significant effect on cell numbers in a single exposure. On hormone production, FB_1 (30 and 100 ng/mL) had no effect on granulosa oestradiol secretion, but significantly stimulated its production at 5 μg/mL. In contrast, α-ZOL (5 μg/mL) and β-ZOL (5 μg/mL) strongly caused a decline in oestradiol production. In addition, a combination of FB_1 (5 μg/mL) with α-ZOL (5 μg/mL) had no effect, but a co-exposure with β-ZOL (5 μg/mL) caused a reduction in oestradiol secretion. Surprisingly, co-treatment of FB_1 alone or in combination with DON, α-ZOL or β-ZOL did not affect bovine granulosa cell progesterone release. In contradiction with the work of Albonico *et al.* (2016), Cortinovis and colleagues reported that co-treatment of FB_1 with α-ZOL amplified the level of progesterone produced by porcine small granulosa cells in single toxin exposure (Cortinovis et al., 2014), indicating additive effects. The difference in the response of porcine and bovine granuolsa cells to co-exposure of FB_1 with α-ZOL

could be as a result of the species sensitivity of these mycotoxins. For instance, it is well known that FB1 is more toxic horses and pigs compared to cattle (Albonico et al., 2016). In an *in vitro* study, Tatay et al. (2014a) studied the cytotoxic and interactive effects of ZEN, α-ZOL and β-ZOL in ovarian (CHO-K1) cells. It was observed that binary or ternary combinations of these mycotoxins showed higher cytotoxicity than the individual toxins. In addition, co-exposure of ZEN with α-ZOL or β-ZOL generally showed additive effects either at 24, 48 or 72 hours of exposure. Interestingly, ternary mixtures of ZEN, α-ZOL and β-ZOL exhibited synergistic effects at lower concentrations (Tatay et al., 2014a), further emphasising the need for the inclusion of mycotoxin mixtures in toxicological assessment. In summary, the co-occurrence of mycotoxins can cause perturbation in cell proliferation and dysregulation of endocrine function of ovarian cells, highlighting the significance of co-occurrence of mycotoxins to female reproductive health. Future research should also examine the role of co-occurrence of mycotoxins in placental morphology and function, embryogenesis and endometrial function. The mechanisms through which mycotoxin mixtures can impact on testicular morphology and steroidogenesis also deserves attention.

In conclusion, mycotoxins are contaminants of many staple food crops for human consumption, particularly in sub-Saharan Africa and South Asia making these toxins a major public health, food safety and economic problem. There is scientific evidence on the adverse reproductive health effects of human exposure to mycotoxins in Africa, including low birth weight, still birth, intrauterine growth restriction, maternal anaemia, neural tube defects and male infertility. However, this review identified papers that were mainly cross-sectional studies and most of the studies did not adjust for other factors that could affect low birth weight, intrauterine growth restriction, male infertility, still birth, and anemia such as malaria, hormonal pathology, malnutrition, poor food quality, and other infectious disease conditions. Therefore, *in vitro* models will be a useful tools in understanding the adverse reproductive and developmental health effects associated with mycotoxins.

At present, the molecular pathways through which mycotoxins induce reproductive and developmental toxicity are still not well understood. Future research should employ validated biomarkers of mycotoxin exposure in human epidemiological studies, and reproductive cell lines or primary cells representing the reproductive system of both

genders to investigate possible molecular pathways in which mycotoxins affect the reproductive systems, the sex gametes, placental and endometrial function, and embryogenesis. In addition, randomised control trials evaluating the effects of mycotoxins and poor reproductive health outcomes in humans using validated exposure biomarkers are urgently needed. Lastly, intervention strategies should be put in place in Africa and Asia to mitigate mycotoxin contamination of food crops, especially those consumed by vulnerable groups such as pregnant women, infants and young children.

Table 1.1: Human studies on mycotoxin exposure, reproductive and developmental toxicity

References	Study design and location	Mycotoxin levels	Outcome	Comments
Abdulrazza et al., 2002	Cord blood samples collected from 201 women at delivery in United Arab Emirates (cross-sectional).	AFM_1 was detected in 107 (53%), AFM_2 in 31 (15%) while AFB_1 in 27 (13%) of the samples. AFB_1: 228 - 15,225 pg/mL; AFM_1: 110 - 4,060 pg/mL; AFM_2: 210 - 3,700 pg/mL	Presence of AFM_1 in cord blood was significantly negatively correlated with birthweight of newborns ($r = 0.63$; $p < 0.001$)	No correlation was found between AFs in cord blood and gestational age.
Abdulrazza et al., 2003	140 breast milk samples from lactating in United Arab Emirates to determine if presence of AFs in breast milk was correlated with gestational age (cross-sectional)	92% of the breast milk samples had detectable AFM_1. Median AFM_1: 560 pg/mL of milk	No significant correlation was found between AFM_1 in breast mik and gestational age, postnatal age, gender and clinical condition.	

References	Study design and location	Mycotoxin levels	Outcome	Comments
Abdulrazza et al., 2004	166 pregnant women and paired cord blood samples from United Arab Emirates to examine if AFM_1 in cord blood was correlated with increased morbidity in newborns (cross-sectional)	AFM_1 was found in 111 (67%) cord and 113 (68%) of maternal blood samples. AFM_1 levels: 0.05 - 10.44 ng/mL (cord blood) and 0.03 - 8.49 ng/mL (maternal blood)	Strong negative correlation was found between AFM_1 levels and birthweight ($r = 0.565$; $p < 0.001$). AFM_1 levels in maternal blood was also strongly correlated with levels in maternal blood samples ($r = 0.797$; $p < 0.00001$)	No association was found bewteen AFM_1 levels in maternal or cord blood samples and rates of jaundice or infection
Abulu et al., 1998	14 cord blood samples from neonates with jaundice and 150 cord blood samples from non-jaundiced neonates to evaluate the	AFs was detected in 81.8 % of cord blood samples in wet season and 50.0% in dry season. Neonates with jaundice had high mean cocentration of AFB_1 (32.3 ng/mL and 35.6 ng/mL).	There was significant decrease in birthweight of jaundiced neonates exposed to AFs ($p < 0.05$).	

References	Study design and location	Mycotoxin levels	Outcome	Comments
	presence of AFs in cord blood in Edo, Nigeria (cross-sectional)			
Ahmed et al., 1995	40 cord blood samples from jaundiced neonates and 37 cord blood samples from non-jaundiced neonates to evaluate the association between perinatal aflatoxin exposure and neonatal jaundice in Zaria, Nigeria (prospective study)	AFs was detected in cord blood of 14 (37.8%) jaundiced neonates and in 9 (22.5%) of non-jaundiced controls. **Jaundiced neonates:** AFB_1: 214 – 238,177 pg/mL; AFB_2: 70 pg/mL; AFM_1: 57 – 713 pg/mL; AFM_2: 374 – 715 pg/mL; AFG_1: 2053 pg/mL; AFG_2: 13 pg/mL; Total AFs: 13 – 238,177 pg/mL **Non-jaundiced neonates:** AFB_1: 474 – 2216 pg/mL; AFM_1: 40 pg/mL; AFM_2:	Average AFs level in cord blood samples was highest in jaundiced neonates with septicaemia but the difference was not statistically significant. No correlation between the severity of hyperbilirubinaemia and cord blood AFs levels was found.	

References	Study design and location	Mycotoxin levels	Outcome	Comments
		32 – 649 pg/mL; AFG_1: 1348 – 1985 pg/mL; AFG_2: 438 pg/mL; Total AFs: 32 – 2654 pg/mL		
Bandera *et al*., 2011	A cross-sectional study to determine the association between the exposure of ZEA and its metabolites on body size and breast development in 163 girls from New Jersey, USA	78.5% of the girls had detectable levels of ZEN and its metabolites (ZEN: 0.05 - 33.12 ng/mL; α-ZOL: 0.003 - 10.69 ng/mL)	ZEN suspected to have anti-oestrogenic effect	Girls exposed to ZEN and its metabolites presented with shortness and had late-onset of breast development

References	Study design and location	Mycotoxin levels	Outcome	Comments
De Vries *et al*., 1989	A cross-sectional study of 125 mother-infant pairs in Kenya	AFs were detected in 53% of maternal blood samples and in 37% of cord samples. **Maternal blood:** AFB_1 (89 – 11,574 pg/mL); AFM_1/AFM_2 (12 – 1,689 pg/mL). **Cord blood:** AFB_1 (86 – 6,819 pg/mL); AFM_1/AFM_2 (17 – 656 pg/mL)	No correlation between AF levels in maternal and cord blood. Female infants born to mothers exposed to high levels AF had significantly lower birth weight compared to those from mothers with non-detectable blood AF.	AF occurrence in maternal and cord blood was significantly higher in the rainy season than in the dry season. Still births occurred in mother and cord bloods with high AFs levels.
Groopman et al., 2014	141 pregnant women with first trimester blood samples and 30 pregnant women with first and third trimester blood samples, both group from Nepal	Aflatoxin B_1-lysine adduct (AFB_1-lys) was present in 132 (94%) maternal blood samples from Nepal and in 63 (100%) maternal and cord blood samples from Bangladesh. AFB_1-lys levels: 0.45 -	AF expsoure was found in pregnancy and children were exposed in the first 1,000 days of life. AF levels were associated with increased levels of alpha-1-acid glycoprotein and C-	

References	Study design and location	Mycotoxin levels	Outcome	Comments
	and 63 pregnant women with first and third trimester bllod samples as well as cord blood samples from Bangladesh (Longitudinal)	2939.30 pg/mg albumin (Nepal); 1.56 - 63.22 pg/mg albumin (Bangladesh - first trimester); 3.37 - 72.8 pg/mg albumin (Bangladesh - third trimester); 4.62 - 76.69 pg/mg albumin (Bangladesh - cord blood samples)	reactive protein during pregnancy	
Ibe *et al*., 1994	Case-control study to determine the association of AFs exposure and male infertility using 50 infertile males and 50 fertile controls from Benin City, Nigeria	The mean AF concentrations was 1.660±0.04 µg/mL in infertile men and 1.041± 0.01 µg/mL in the fertile control group). Aflatoxins were detected in 40% of the infertile men compared to 8% in Fertile controls	AF exposure was associated with increased sperm abnormality	Higher percentage of sperm abnormality (50.0%) was found in infertile males with high exposure to AFs compared to the fertile control group (10.0-15.0%)

References	Study design and location	Mycotoxin levels	Outcome	Comments
Jonsyn et al., 1995	64 cord blood and 8 maternal blood samples collected at delivery to examine in utero transfer of AFs and Ochratoxin A (OTA) in Sierra Leone	AFs was detected in 58 (90.6%) of cord blood samples whereas OTA was found in 16 (25%). **Mycotoxin levels in cord blood:** OTA: 0. 2 – 3.5 ng/mL; AFB_1: 0.4 – 9.0 ng/mL; AFB_2: 0.02 – 1.2 ng/mL; AFM_1: 0.007 – 5.1 ng/mL; AFM_2: 0.02 – 5.4 ng/mL; AFG_1: 0.004 – 8.8 ng/mL; AFG_2: 0.002 – 3.0 ng/mL; AFL: 0.007 – 2.2 ng/mL	No relationship between mycotoxin levels in maternal and cord blood. Girl neonates exposed to OTA and AFs in utero had lower birthweights ($p < 0.05$)	
Lamplugh *et al*., 1988	Blood samples from 18 cord bloods and 264 breast milk samples from Ghana and 77 mother-infants	AFs detected in 64 (34%) cord bloods and 90 (34%) breast milk from Ghana, and 16 maternal (21%) and 9 (12%) cord blood from Nigeria.	AF occurrence and concentration was detected more in the rainy season than in dry season. Presence of AF biomeasures in cord blood confirmed AF	One stillbirth was recorded from a mother from high AFs exposure level (AFB_1: 553 ng/L)

References	Study design and location	Mycotoxin levels	Outcome	Comments
	pairs from Nigeria with maternal and cord blood	**Ghana cord:** AFB_1 (185 – 43,822 ng/L); AFB_2 (11 – 925 ng/L); AFM_1 (34 – 7,320 ng/L); AFM_2 (30 – 572 ng/L); AFG_1 354 -1354 ng/L), AFG_2 (37 ng/L); AFL (117 ng/L) **Ghana breast milk:** AFB_1 (130 – 8218 ng/L); AFB_2 (49-50 ng/L); AFL (64-270 ng/L); AFM_1 (20 – 1,816 ng/L); AFM_2 (16 – 2,075 ng/L) **Nigeria maternal blood:** AFB_1 (540 – 10,390 ng/L); AFB_2 (28 – 33 ng/L); AFM_1 (38 – 483 ng/L); AFM_2 (48 – 3,480 ng/L) Nigeria cord blood: AFB_2 (0 - 10 ng/L); AFM_1 (25 – 8,942 ng/L); AFM_2 (208 –	crosses placental barrier to the foetus.	

References	Study design and location	Mycotoxin levels	Outcome	Comments
		378 ng/L)		
Massart *et al*., 2008	Study on serum samples of 32 girls with central precocious puberty (CPP) and 31 female healthy female controls from North-West Tuscany, Italy	ZEN (723.5 - 1143.9 pg/mL) and α-ZOL (104.5 - 108.5 pg/mL) were detected from the serum of 6 of the 32 girls with CPP. All the controls had no detectable mycotoxins	ZEN suspected of inducing central maturation of the hypothalamic-pituitary-gonadal (HPG) axis causing CPP in exposed girls	Mycotoxin positive patients had higher height, weight and height velocity compared to those who had no mycotoxin detected in their serum

References	Study design and location	Mycotoxin levels	Outcome	Comments
Maxwell *et al*., 1994	A cross-sectional study to evaluate the effect of AFs and napthols exposure on birth weight of 625 babies at delivery in Ibadan, Nigeria.	AFs were found in 91 (14.6%) of the cord bloods. The distributions and levels were: AFB_1 (168 – 69,973 ng/mL); AFB_2 (15 – 144 ng/mL); AFM_1 (32 – 11,354 ng/mL); AFM_2 (14 – 3,644 ng/mL)	*In utero* AF and napthols exposure did not correlate with birth weight at delivery.	
Mohammed *et al*., 2014	A case-control hospital-based study that examined the AFs concentration in semen samples from 108 (60 infertile men and 48 fertile controls) visiting Sohag University Hospital, Egypt	AFB_1 was present in 25% of the semen of infertile patients, compared to 2.1% among the controls (P=0.0007)	AFB_1 exposure was suggested as a potential contributor to idiopathic infertility in males.	Severe reduction in sperm count, reduced motility, high Percentage of sperm with abnormal morphology, and high viscosity in the semen of the infertile group($P<0.05$) compared to the fertile group

References	Study design and location	Mycotoxin levels	Outcome	Comments
Sedeghi et al., 2009	160 breast milk samples from lactating mothers in Thran, Iran to examine the exposure of infants to AFM_1 and of lactating mothers to AFB_1 (cross-sectional), using AFM_1 in breast milk as biomarker of AFB_1 exposure	AFM_1 was detected in 157 (98.1%) breast milk samples ranging 0.3 to 26.7 ng/kg	TThere was significant association between AFM_1 levels in breast milk and height of infants at birth ($p < 0.01$)	No significant correlation was found between AFM_1 levels in breast milk and gestational age, post-ntal age and clinical condition
Shuaib *et al*., 2010b	Blood from 755 pregnant Ghanaian women	Blood AF-lysine adduct detected by HPLC and all the blood samples had detectable AF-lysine adduct (range: 0.44 – 268.73 pg/mg albumin)	Pregnant women with AF-lysine in the highest quartile (> 11.34 pg/mg) were 2.09 times more likely to have low birthweight infants (95% CI: 1.19 – 3.68)	Other parameters showed a trend towards an increased risk (P_{trend} = 0.007)

References	Study design and location	Mycotoxin levels	Outcome	Comments
Shuaib *et al*., 2010c	Blood from 755 pregnant Ghanaian women	Blood AF-lysine adduct detected by HPLC and all the blood samples had detectable AF-lysine adduct (range: 0.44 – 268.73 pg/mg albumin; Mean: 10.9± 19.00 pg/mg albumin). 30.3% had anaemia	Very high blood AF-lysine adduct was significantly associated with the odds of being anaemic (OR: 1.85; CI: 1.16 – 2.95)	The odds of being anaemic was stronger in pregnant women after exclusion of those with malaria, intestinal parasitic infection or low folate level. AF exposure in pregnancy was associated with anaemia.
Sodeinde et al., 1995	Blood samples were collected from 327 jaundiced neonates and 80 of their mothers, and 60 non-jaundiced neonates and seven of their mothers in Ibadan, Nigeria to investigate the	AFs were found in 74 (27.4%) of jaundiced neonates, 12 (17%) of their mothers, 10 (16.6%) of non-jaundiced controls and 1 (14.4%) of control mothers. **Jaundiced groups:** AFB_1 (256 - 58,239 ng/L); AFB_2 (5 - 17 ng/L); AFM_1 (48 - 32,381 ng/L); AFM_2 (407 - 9280 ng/L); AFL (26 - 750	Serun AFs is a risk factor for neonatal jaundice (OR: 2.68; CI: 1.18 - 6.10)	

References	Study design and location	Mycotoxin levels	Outcome	Comments
	prevalence of naphtols and AFs in the sera of babies with neonatal jaundice and their mothers in order to assess if they contribute to unexplained neonatal jaundice in Ibadan (cross sectional)	ng/L); AFG_1 (460 - 165,067 ng/L); AFG_2 (21 - 990 ng/L) **Non-jaundiced control infants:** AFB_1 (1956 - 20,371 ng/L); AFM_1 (80 - 11,547 ng/L); AFM_2 (3262 4350 ng/L); AFG_1 (1112 - 4370 ng/L); AFG_2 (70 ng/L)		
Szuets *et al*., 1997	Study on young girls with early thelarche/mastopathy in Southern Hungary	ZEN was detected in the serum of 5 out of 36 early patients with concentrations ranging from 18.9 - 103 µg/mL	ZEN reported to be associated with early thelarche/mastopathy	Childhood ZEN exposure was associated with early breast development in young girls

References	Study design and location	Mycotoxin levels	Outcome	Comments
Tomaszewski *et al*., 1998	Endometrial tissue specimens collected from 49 women (endometrial adenocarcinoma n = 27, endometrial hyperplasia n = 11, normal proliferative endometrium n = 11).	The concentration of ZEN in 3 endometrial hyperplasia samples was 47.8±6.5 ng/ml and 167.0±17.7 ng/ml in 22 adenocarcinoma samples	ZEN suspected to be associated with hyperplastic and neoplastic endometrium	Normal endometrial samples had no detectable concentration of ZEN. Also 8 hyperplastic and 5 neoplastic endometrial samples had no detectable concentration of ZEN
Turner *et al*., 2007	Maternal and cord blood was collected from 138 singleton infants followed for 14 months in The Gambia	AF-alb adducts detected by ELISA and 119 (100%) maternal blood, 48 (48.5%) cord blood and 13 blood samples (11%) from week 16 children had detectable AF-alb adducts. **Maternal:** 23.3-64.1 pg/mg albumin (Mean:	Higher AF-alb levels in maternal blood (at 5 and 8 months of pregnancy) were associated with lower HAZ (-0.207 SD; $P = 0.044$) and WAZ (-0.247 SD; $P = 0.012$) scores in children during the first year of life.	There was seasonal variation in maternal AF-alb levels. Maternal AF-alb levels was significantly higher in blood samples collected in December – March compared to samples collected in either April – November ($P < 0.001$)

References	Study design and location	Mycotoxin levels	Outcome	Comments
		38.9 pg/mg albumin); **Cord:** 2.5-7.9 pg/mg albumin (Mean: 2.5 mg/mg albumin)	Maternal AF-alb in pregnancy was not associated with infant weight or length at birth	
Uriah *et al*., 2001	A case-control study to examine the association between aflatoxin exposure and human reproduction in Benin City, Nigeria using 30 infertile males and 25 fertile controls	Serum and semen AFB_1 concentrations ranged from 700 - 1392 ng/mL and 60 - 148 ng/mL, respectively in infertile males with values significantly higher than those in the fertile group. One semen sample with no live spermatozoa (azoospermia) had an AFB_1 levels as high as 1450 ng/mL. Approximately 37% of semen and blood of infertile men had detectable aflatoxins than the controls	AF exposure suggested as a contributory factor to male infertility in Nigeria	AFs exposure caused decreased sperm concentration and increased sperm abnormalities

Key: CI: confidence interval; **OR:** odd ration; **SD:** standard deviation; **HAZ:** height-for-age Z-score; **WAZ:** weight-for-height Z-score; **HPLC:** high performance liquid chromatography; **AFL:** aflatoxicol; **AF:** aflatoxin; **AFs:** AFB_1, AFB_2, AFM_1, AFM_2, AFG_1, and AFG_2; **ZEA:** zearalenone; **α-ZOL:** α-zearalenol; **ELISA** = Enzyme-linked immunosorbent assay; **OTA:** ochratoxin A

Table 1.2: Occurrence and concentrations of OTA (ng/mL) in human serum, breast milk and urine from different countries

Reference	Country of study	Sample type	Sample size (N)	Incidence (%)	Range (ng/mL)
Abid et al., 2003	Tunisia	Blood	CIN: 954 Healthy subjects: 205	CIN: 93-100% Healthy subjects: 62-82%	CIN: 23 - 66.2 Healthy subjects: 0.1 - 2.3
Aslam et al., 2005	Pakistan	Blood	31	97	0.04 - 1.24
Breitholtz et al., 1991	Sweden	Blood	297	13	0.3 - 6.7
Breitholtz-Emmanuelsson et al., 1994	Italy	Blood	65	100	0.12 - 2.0
Coronel et al., 2009	Spain	Blood	279	99	0.11 - 8.68
Coronel et al., 2011	Spain	Urine	72	13	0.057 - 0.562
Creppy et al., 1993; Benford et al., 2001	France	Blood	3,070	18	≤161.0

Reference	Country of study	Sample type	Sample size (N)	Incidence (%)	Range (ng/mL)
Domijan et al., 2003	Croatia	Urine	35	94	0.99 - 5.22
Duarte et al., 2010	Portugal	Urine	30	93	n.d - 0.069
El-Sayed et al., 2000	Egypt	Breast milk	10	30	3.0 - 15.0
El-Sayed et al., 2002	Egypt	Breast milk	120	36	5.1 - 45.0
Erkekoglu et al., 2010	Turkey	Blood	Black sea (summer): 60 Black sea (winter): 59 Mediterranean (summer): 59 Mediterranean (winter): 61	Black sea (summer): 98 Black sea (winter): 81 Mediterranean (summer): 97 Mediterranean (winter): 72	Black sea (summer): 0.043 - 1.496 Black sea (winter): 0.031 - 0.887 Mediterranean (summer): 0.028 - 1.398 Mediterranean (winter): 0.035 - 0.707
Filali et al., 2002	Morocco	Blood	309	60	0.08 - 6.59

Reference	Country of study	Sample type	Sample size (N)	Incidence (%)	Range (ng/mL)
Fuchs et al., 1991	Former Yugoslavia	Blood	3,378	3.7	0 - 50
Ghali et al., 2008	Tunisia	Blood	40	28	0.1 - 11.98
Gilbert et al., 2001	UK	Urine	50	92	0.01 - 0.058
Goslinki et al., 1991	Poland	Blood	1,065	7.2	0.27 - 40
Grosso et al., 2003	Tunisia	Blood	62	100	0.12 - 8.06
Gurbay et al., 2010	Turkey	Breast milk	75	100	0.62 - 13.11
Haldi, 1991	Denmark	Blood	144	54	0.10 - 13.2
Hassen et al., 2004	Tunisia	Blood	N/A	CIN patients: 93 Nephro patients: 83 CIN UA patients: 100 CIN KA patients: 78	CIN patients: ≤140.5 Nephro patients: ≤73.2 CIN UA patients: 18.4 -171.3 CIN KA patients: ≤29.0
Jimenez et al., 1998	Spain	Blood	75	59	0.52 - 4.0

Reference	Country of study	Sample type	Sample size (N)	Incidence (%)	Range (ng/mL)
Johnsyn et al, 1999	Sierra Leone	Urine	54	24	0.3 - 26.6
Johnsyn et al, 2001	Sierra Leone	Urine	Mokonde: 244 (dry season); 190 (rainy season)	Dry season: 26; Rainy season: 25	Dry season: 0.07 - 148; Rainy season: 0.6 - 72.2
Johnsyn-Ellis, 2007	Sierra Leone	Blood	131	19	0.4 - 60
Jonsyn et al., 1995b	Sierra Leone	Breast milk	113	35	0.2 - 337.0
Jonsyn et al., 1999	Sierra Leone	Breast milk	31	30	n.d - 39.4
Karima et al., 2010	Tunisia	Blood	105	28	0.12 - 3.43
Kovacs et al., 1995	Hungary	Breast milk	92	41	0.22 - 7.63
Malir et al., 2001	Czech Republic	Blood	809	91	0.1 - 13.7
Malir et al., 2006	Czech Republic	Blood	2206	94	0.1 - 13.7
Manique et al., 2008	Portugal	Urine	30	43	0.011 - 0.208
Medina et al., 2010	Spain	Blood	168	100	0.15 - 5.71
Micco et al., 1991	Italy	Breast milk	50	18	1.2 - 6.6
Micco et al., 1995	Italy	Breast milk	111	20	0.1 - 12
Munoz et al. 2010a	Chile	Breast milk	9	100	0.044 - 0.184
Munoz et al., 2006	Chile	Blood	Colbun: 44	Colbun: 54	Colbun: 0.07 -2.75

Reference	Country of study	Sample type	Sample size (N)	Incidence (%)	Range (ng/mL)
			Tagua: 44	Tagua: 91	Tagua: 0.22 -2.12
Munoz et al., 2010b	Germany	Urine	13	100	0.02 - 0.14
Pacin et al., 2008	Argentina	Blood	Madel Plata: 205 General Rodrubuez: 275	Madel Plata: 64 General Rodrubuez: 62	Madel Plata: 0.01 - 47.6 General Rodrubuez: 0.019 - 74.8
Palli et al., 1999	Italy	Blood	138	97	0.12 - 57.2
Perez de Obanos et al., 2001	Spain	Blood	83	87	0.22 - 6.96
Petkova-Bocharova et al., 2003	Bulgaria	Urine	55	87	0.01 - 1.91
Rosner et al., 2000	Germany	Blood	927	98	0.06 - 2.03
Ruprich and Ostry, 1993a	Czech Republic	Blood	594	40	0.05 - 37.0
Ruprich and Ostry, 1993b	Czech Republic	Blood	644	22	n.d - 12.0
Scott et al., 1998	Canada	Blood	144	100	0.29 - 2.37
Skaug et al., 2003	Norway	Blood	210	100	0.021 - 5.534

Reference	Country of study	Sample type	Sample size (N)	Incidence (%)	Range (ng/mL)
Ueno et al., 1998	Japan	Blood	184	85	0.004 - 0.278
Wafa et al., 1998	Egypt	Urine	111	17	0 - 8.19
Zimmerli and Dick, 1995	Switzerland	Blood	368	100	0.06 - 6.02
Zimmerli and Dock, 1995	Switzerland	Breast milk	40	10	0.01 - 5.0

CIN Patients: Patients with chronic interstitial nephropathy (CIN) of unknown etiology; **Nephro patients:** Chronic nephropathies of known etiologies; **CIN UA:** CIN of unknown etiology; **CIN KA:** CIN of known etiology; n.d: non-detectable; **N/A:** Not available

Table 1.3: Occurrence and concentrations of DON (ng/mL) in the general population

Reference	Country of study	Sample type	Sample size (N)	Incidence (%)	Range
Abia et al., 2013	Cameroon	urine	145	43	≤10 - 74.7
Ali et al., 2015	Bangladesh (pregnant women)	urine	54	52	n.d - 7.16
Ali et al., 2016	Bangladesh (adults)	urine	Summer: 62 Winter: 62	Summer: 27 Winter: 31	Summer: n.d -1.78 Winter: n.d -1.21
Ali et al., 2016	German (adults)	urine	50	100	1.06 - 38.44
Ediage et al., 2012	Belgium (adults)	urine	40	13	≤0.7 - 68.3
Ediage et al., 2013	Cameroon (children)	urine	220	73	0.1 - 77.0
Ezekiel et al., 2014	Nigeria (adolescents, children and adults)	urine	120	5	Total DON: <LOQ - 10.0
Föllmann et al., 2016	Germany (adults)	urine	30	100	0.85 - 14.63
Gerding et al., 2014	Germany (adults)	urine	101	29	2.48 - 17.34
Gerding et al., 2015	Haiti (adults and	urine	142	17	<LOQ - 16.9

Reference	Country of study	Sample type	Sample size (N)	Incidence (%)	Range
	children)				
Gerding et al., 2015	Germany (adults)	urine	50	16	Mean: 2.0±n.a
Gratz et al., 2014	UK (Adults)	urine	Year 1: 15 Year 2: 15	Year 1: 100 Year 2: 100	Year 1: 0.3 -27.5 Year 2: 0.03 -28.6
Hepworth et al., 2012	UK (Pregnant women)	urine	85	100	0.5 - 116.7
Heyndrickx et al., 2015	Belgium (adults, children)	urine	Adults: 239 Children: 155	Adults: 37 Children: 70	Adults: 0.5 - 129.8 Children: 0.5 - 32.5
Huybrechts et al., 2015	Belgium	urine	32	100	DON-15GlcA: ≤3.0 - 420
Kouadio et al., 2014	Ivory Coast (adults)	urine	99	21	n.d - 10.0
Meky et al., 2003	China	urine	High-risk area: 11 Low-risk area: 4	High-risk and Low-risk area: 100	High-risk area: 4.0 - 94.0 Low-risk area: 4.0 - 18.0

Reference	Country of study	Sample type	Sample size (N)	Incidence (%)	Range
Rodriguez-Carrasco et al., 2014	Spain (children and adults)	urine	54	69	1.6 - 30.4
Sarkanj et al., 2013	Croatia (pregnant women)	urine	40	76	DON: n.d - 275 DON-15-GlcA: n.d - 1238 DON-3-GlcA: n.d - 298
Shephard et al., 2013	South Africa	urine	54	87	≤0.5 - 53.4
Solfrizzo et al., 2011	Italy (adults)	urine	10	70	<0.8 - 14.2
Solfrizzo et al., 2014	Italy (adults)	urine	52	96	n.d - 67.36
Turner et al., 2008a	UK	urine	25	100	0.7 - 61.3
Turner et al., 2010a	France (adults)	urine	76	99	0.5 - 28.8
Turner et al., 2010b	UK (Adults)	urine	35	100	0.5 - 78.2
Turner et al., 2011a	China (women)	urine	60	97	n.d - 29.9
Turner et al., 2011b	UK (Adults)	urine	34	68	0.5 - 9.3
Wallin et al., 2013	Sweden (adults)	urine	326	90	<0.5 - 65.8
Wallin et al., 2015	Sweden (adults)	urine	252	63	Mean: 5.3±12.3
Warth et al., 2012	Austria (Adults)	urine	27	59	n.d - 61

Reference	Country of study	Sample type	Sample size (N)	Incidence (%)	Range
Wells et al., 2016	UK (pregnant women)	urine	42	Day 1: 88 Day 2: 83	Day 1: 0 - 436 Day 2: 0 - 167

Table 1.4: Occurrence and concentrations of AFB_1 in the general population

Reference	Country of study	Sample type	Sample size (N)	Incidence (%)	Range
Abdularazzaq et al., 2002	United Arab Emirates	Cord blood	201	13	228 - 15,225 pg/ml
De Vries et al., 1989	Kenya	blood	125 maternal and cord blood	Maternal: 53 Cord: 37	Maternal: 89 – 11,574 pg/ml Cord: 86 - 6,819 pg/ml
Ibeh et al. 1994	Nigeria	Semen	Fertile: 50 Infertile: 50	Fertile: 40 Infertile: 8	Infertile: 1,660 ng/mL Fertile: 1,041ng/mL
Jonsyn-Ellis, 2001	Sierra Leone	Urine	Boys: 134 Girls: 110	Boys (rainy season): 33 Boys (dry season): 35 Girls (rainy season): 41	Boys (rainy season): 1.2 - 115 ng/ml Boys (dry season): 0.6 - 188ng/ml Girls (rainy season): 0.08 - 127 ng/ml Girls (dry season): 0.04 - 319 ng/ml

Reference	Country of study	Sample type	Sample size (N)	Incidence (%)	Range
				Girls (dry season): 49	
Jonsyn-Ellis, 2007	Gambia	blood	n.a	n.a	0.2 - 74 ng/ml
Lamplugh et al., 1988	Ghana	Cord blood and breast milk	Cord blood: 188 Breast milk: 264	9	Cord: 185 - 43,822 ng/L Breast milk: 130 – 1816 ng/l
Lamplugh et al., 1988	Nigeria	Maternal and cord blood	77	Maternal: 77 Cord: 12	Maternal: 553 – 10,390 ng/l Cord: Nil
Maxwell et al., 1994	Nigeria	blood	625 infants	14.6	168 - 69,973 ng/L
Polychronaki et al., 2008	Guinea	urine	50	16	Mean: 2682 pg/ml (range: 179 – 18,000 pg/ml)
Polychronaki et al., 2008	Egypt	urine	50	2	Mean: 189 pg/ml
Uriah et al., 2001	Nigeria	Blood	Fertile: 25 Infertile: 30	Fertile: 16 Infertile: 37	Fertile: 5 - 20 ng/mL Infertile: 170 - 350 ng/ml
Uriah et al., 2001	Nigeria	Semen	Fertile: 25 Infertile: 30	Fertile: 8 Infertile: 37	Fertile: 0 - 5 ng/ml Infertile: 60 - 460 ng/ml

Table 1.5: Occurrence and concentrations (ng/mL) of ZEN and metabolites in the general population

Reference	Country of study	Sample type	Sample size (N)	Incidence (%)	Range
Abia et al., 2013	Cameroon	Urine	145	5	Total ZEN: ≤LOQ - 21.4
					Free ZEN: ≤LOQ - 1.42
					ZEN-14-GlcA: 3.38 - 31
Bandera et al., 2014	USA	Urine	163	55	ZEN: ≤0.05 - 33.1
					α-ZOL: 0.003 - 10.69
Belhassen et a., 2015	Tunisia	Urine	69 breast cancer cases; 41 controls	30.9%	α-ZAL (Breast cancer cases): 0.7 - 11.8
					(Mean: 3.9)
					α-ZAL (Healthy controls): N/A (Mean: 2.9)
Ediage et al., 2012	Belgium	Urine	40	10	ZEN: ≤1.2 - 12.6
Ediage et al., 2013	Cameroon	Urine	220	ZEN: 3.6	ZEN: 0.65 - 5.0
				α-ZOL: 4	α-ZOL: 0.26 - 1.3
				β-ZOL: 8	β-ZOL: 0.02 - 12.5
Ezekiel et al., 2014	Nigeria	Urine	120	Total ZEN: 7	Total ZEN: LOQ - 44.5
Gerding et al., 2015	Haiti	Urine	142	3	α-ZOL: 0.52 - 2.49

Reference	Country of study	Sample type	Sample size (N)	Incidence (%)	Range
Heyndrickx et al., 2015	Belgium	Urine	239	0.2	α-ZOL: 5.0 - 5.0
Huybrechts et al., 2015	Belgium	Urine	32		ZEN: ≤0.05
Massart et al., 2008	Italy	Blood	32 girls with central precocious puberty and 31 female healthy female controls	18.75	ZEN: 723.5 - 1143.9 pg/mL α-ZOL: 104.5 - 108.5 pg/mL
Pillay et al., 2002	South Africa	Blood	132	N/A	ZEN: 200 – 457 α-ZOL: 70 – 378 β-ZOL: 110 – 707
Shephard et al., 2013	South Africa	Urine	54	ZEN: 100 α-ZOL: 92 β-ZOL: 75	ZEN: 0.012 - 3.5 α-ZOL: 0.009 - 3.72 β-ZOL: 0.016 - 5.94
Solfrizzo et al., 2014	Italy	Urine	52	ZEN: 100 α-ZOL: 100 β-ZOL: 98	ZEN: LOQ - 0.120 α-ZOL: LOQ - 0.176 β-ZOL: LOQ - 0.135

Reference	Country of study	Sample type	Sample size (N)	Incidence (%)	Range
Suetz et al., 1997	Southern Hungary	blood	36	14	ZEN: 18.9 - 103
Tomaszewski et al., 1998	N/A	Endometrial tissues	49 endometrial tissues (endometrial adenocarcinoma n = 27, endometrial hyperplasia n = 11, normal proliferative endometrium n = 11)	N/A	ZEN (3 endometrial hyperplasia): 47.8±6.5 ZEN (22 adenocarcinoma samples): 167.0±17.7
Wallin et al., 2015	Sweden	Urine	252	ZEN: 37 α-ZOL: 21 β-ZOL: 18	ZEN: 0.007 - 0.42 α-ZOL: 0.029 - 1.83 β-ZOL: 0.054 - 1.33

Table 1.6: Summary of selected studies on human exposure to mycotoxin mixtures[#]

References	Country	No. of subjects	Individuals examined	No of positive samples (%)	Mycotoxin exposure biomarkers detected	Co-exposure in a single individual
Abia *et al*., (2013)	Cameroon**		145 HIV-positive adults (29 male, 116 female), 30 HIV-negative adults	110 (63%)	DON, NIV, ZEA, OTA, FB_1, FB_2, DON-15-GlcA, DON-3-GlcA, ZEA-14-GlcA, α-ZOL, AFM_1	DON, OTA, NIV, FB_1, ZEA, AFM_1, FB_2
Ahn *et al.*, (2010)	Korea**	12	11 adults, 1 child	12 (100%)	AFM_1, OTA	AFM_1, OTA
Cao *et al*., (2013)	China*	10	6 men, 1 pregnant woman, 3 lactating women	Not stated	AFB_1, AFB_2, HT-2, DON, DOM-1, ZEA, α-ZOL, β-ZOL, FB_1, FB_2, AFM_1, OTA, NEO, T-2 Triol,	3 samples had AFBs and FBs.
Ediage *et al*., (2012)	Belgium**	40	Adults	9 (23%)	DON, OTA, OTα, 4-OH-OTA, ZEA, CIT, β-ZOL	DON, OTA, OTα, ZEA, β-ZOL
Ediage *et al*., (2013)	Cameroon**	220	220 children	160 (73%)	OTA, DON, AFM_1, FB_1, ZEA, α-ZOL, β-ZOL	Co-occurrence of 2, 3 and 4 mycotoxins was 35%, 5% and 5%, respectively.
Ezekiel *et al*., (2014)	Nigeria**	120	81 adults, 20 adolescents, 19 children	61 (50.8%)	AFM_1, DON, DON-15-GlcA, FB_1, FB_2, OTA, ZEA, ZEA-14-GlcA	Distribution of mycotoxins: 75% (46/61) had a single mycotoxin and 25% (15/61) had more than 1 mycotoxin [8 had 2 different mycotoxins, 5

References	Country	No. of subjects	Individuals examined	No of positive samples (%)	Mycotoxin exposure biomarkers detected	Co-exposure in a single individual
						had 3 different mycotoxins, 2 had 4 different mycotoxins, 7 had more than one mycotoxin/metabolite].
Gerding *et al*., (2014)	Germany**	101	Adult volunteers	87%	DON, ZEA, CIT, T-2, ENNB, DON-3-GlcA, ZEA-14-GlcA, DH-CIT	DON-ENNB-ZEA, DON-CIT-T-2, DON-CIT, DON-ZEA-DON-ENNB
Gerding *et al*., (2015)	Bangladish**, Germany**, Haiti**	287	95 adult Bangladeshis, 50 adult Germans, 142 adult Haitians	Bangladish (87%), Germany (80%), Haiti (68%)	DON, OTA, CIT, ENNB, AFM_1, FB1, α-ZOL, DON-3-GlcA, DH-CIT	DON-CIT-OTA-FB_1, DON-CIT-OTA, DON-OTA-ENNB, CIT-OTA-ENNB, CIT-OTA-FB1, AFM_1-CIT-OTA, AFM_1-CIT-DON, ENNB-OTA, DON-OTA, CIT-OTA, CIT-FB_1, CIT-ENNB, AFM_1-CIT
Rodriguez-Carrasco *et al.*, (2014)	Valencia**	54	38 adults, 16 children	37 (68.5%)	HT-2, NIV, DON	DON-HT2, DON-NIV
Rubert *et al.*, (2011)	Spain**	27	Adults	Not stated	AFG_2, OTA, DON	Not stated
Shephard *et al*., (2013)	South Africa**	53	Adult women	53 (100%)	OTA, FB_1, DON, DON-3-GlcA, DON-15-GlcA, NIV, ZEA, ZEA-14-GlcA, α-ZOL, β-ZOL	OTA, FB_1, DON, DON-3-GlcA, DON-15-GlcA, ZEN, ZEA-14-GlcA, α-ZOL, β-ZOL

References	Country	No. of subjects	Individuals examined	No of positive samples (%)	Mycotoxin exposure biomarkers detected	Co-exposure in a single individual
Solfrizzo *et al.*, (2011)	Italy**	10	Adults	10 (100%)	OTA, DON	DON, OTA
Solfrizzo *et al.*, (2014)	Italy**	52	26 males, 26 females	52 (100%)	DON, OTA, AFM_1, FB_1, ZEA, α-ZOL, β-ZOL	Distribution of Mycotoxin mixtures in samples: 2 (DON, ZEA, FB_1, OTA, AFM_1); 27 (DON, ZEA, FB1, OTA); 20 (DON, ZEA, OTA); 1 (DON, ZEA, OTA, AFM_1); 2 (ZEA and OTA).
Warth *et al.*, (2012a)	Austria**	27	Adults	26 (96%)	DON, DON-3-GlcA, DON-15-GlcA	DON, DON-3-GlcA, DON-15-GlcA
Warth *et al.*, (2012b)	Cameroon**	175	145 HIV-positive adults, 30 HIV-negative adults	110 (63%)	AFM_1, OTA, FB_1, FB_2, DON, DON-3-GlcA, DON-15-GlcA, NIV, ZEA, ZEA-14-GlcA, α-ZOL	AFM_1, OTA, FB_1, FB_2, DON, DON-3-GlcA, DON-15-GlcA, NIV

[#] These are human mycotoxin exposure studies reporting two or more mycotoxins in single sample using LC-MS/MS multi-mycotoxin method and conducted between 2010 and 2015; *Urine, faeces, breast milk and amniotic fluid were analysed. **Only urine samples were analysed.
Abbreviations used: NEO - Neosolaniol; AFB_1 = Aflatoxin B_1; DON = Deoxynivalenol; ZEA = Zearalenone; OTA = Ochratoxin A; OTα = Ochratoxin alpha; 4-OH-OTA = 4-hydoxyl-ochratoxin A; NIV = Nivalenol; AFM_1 = Aflatoxin M_1; AFG_2 = Aflatoxin G_2; FB_1 = Fumonisin B_1; FB_2 = Fumonisin B_2; α - and β-ZOL = α - and β-Zearalenol; ZEN-14-GlcA = Zeralenone-14-O-glucuronide; α-ZOL-14-GlcA = α-Zearalenol-14-O-glucuronide; α-ZOL-7-GlcA = α-Zearalenol-7-O-glucuronide; β-ZOL-14-GlcA = β-Zearalenol-14-O-glucuronide; β-ZOL-16-GlcA = β-Zearalenol-16-O-glucuronide; DON-15-GlcA = Deoxynivalenol-15-glucuronide; DON-3-GlcA = Deoxynivalenol-3-glucuronide; DOM-1 = de-epoxy-deoxynivalenol; CIT = Citrinin; ENNB = Enniatin B; DH-CIT = Dihydrocitrinone; HIV = Human immunodeficiency virus

1.3 Persistent organochloride pesticides and reproductive health

The persistent organochloride pesticides (POPs) are the most commonly used insecticides and contain carbon, hydrogen and chlorine moieties in their molecular structure. Most of these chemicals are lipophilic and non-biodegradable and therefore, persist in the environment, bioaccumulate in fatty tissues and are biomagnified through the food chain. Among the POPs, dichlorodiphenyltrichloroethane (DDT) and its isomers and metabolites have been of major public health concern due to their adverse effects on reproductive health (ATSDR, 2002). Technical DDT used as pesticide/insecticide contains a mixture of 1, 1, 1-trichloro-2,2-bis (*p*-chlorophenyl)-ethane (*p,p′*-DDT; 85%), 1, 1, 1-trichloro-2-(*0*- chlorophenyl)-2-(*p*-chlorophenyl)-ethane (*o,p′*-DDT; 15 – 21%), and trace amounts of of other components (ATSDR, 2002). In the biological system and the environment, *p,p′*-DDT (**Fig. 1.7**) is metabolised to its major metabolite 1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethylene (*p,p′*-DDE) which has a longer half life compared to *p,p′*-DDT (Bornman et al., 2018). Although the use of DDT is banned in Europe and North America, it is still used in developing countries, especially in Africa for the control of mosquitoes through long lasting DDT treated bed nets or indoor residual spraying programmes. Noteworthy is the illegal diversion to agricultural use which remains a major concern in developing countires in Africa and Southern Asia.

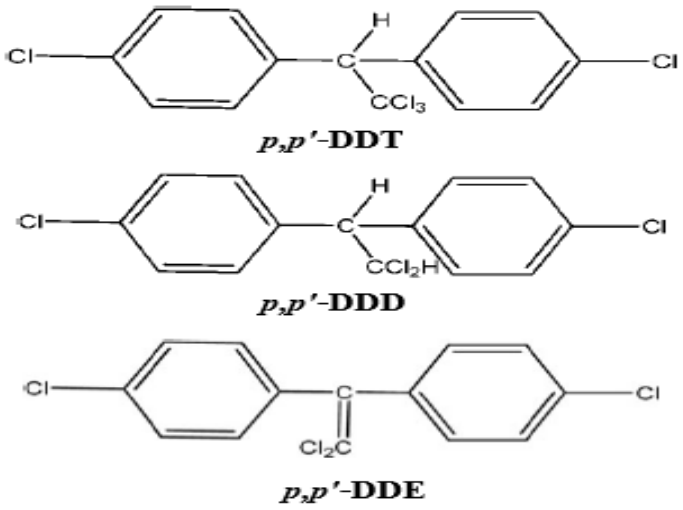

Fig. 1.7: The chemical structure of 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane (*p,p′*-DDT) and its major metabolites 1,1-dichloro-2,2-bis(p-chlorophenyl) ethylene (*p,p′*-DDE) and 1,1-dichloro-2,2-bis(4-chlorophenyl)ethane (*p,p′*-DDD) (Source: Zhuang et al., 2012; Reproduced with permission from the publisher John Wiley & Sons, Inc.)

In adults, exposure to DDT is mainly through dietary intake, although inhalation and skin contact also contribute to DDT burden. DDT can accumulate in the placenta and cross the placental barrier, and therefore, prenatal exposure can occur *in utero*. In addition, it is commonly excreted in breast milk of exposed women and early life exposure occurs through breast-feeding (Mrema et al., 2013). The lipophilic nature of *p,p′*-DDT and its major metabolite *p,p′*-DDE makes them bioaccumulate in fatty tissues in the human body and have been detected in breast milk, blood plasma/serum, placenta, cord blood, adipose tissues and follicular fluids (Rogan and Chen, 2005; Jirsová et al., 2010; Dewan et al., 2013). Both *p,p′*-DDT and *p,p′*-DDE are oestrogen receptor agonists whereas *p,p′*-DDE is also a potent androgen receptor antagonist (Kelce et al., 1995). Several studies have reported the adverse effects of *p,p′*-DDT and *p,p′*-DDE on human reproduction (**Table 1.7**). However, reports from human epidemiological studies are inconsistent and the comparison of findings is hampered by differences in exposure concentrations, duration of exposure, samples type and study population (Bornman et al., 2018). Several studies have revealed the negative effects of *p,p′*-DDT exposure on male reproductive hormones and semen quality. For instance, in Mexican men involved in indoor residual spray of DDT, serum *p,p′*-DDE was significantly correlated with reduced semen volume, sperm count and the ratio of bioavailable to total testosterone level (Ayotte et al., 2001). Similarly, *p,p′*-DDT and *p,p′*-DDE exposure was associated with poor semen quality (Aneck-Hahn et al., 2007) and negatively correlated with serum testosterone and oestradiol levels (Asawasinopon et al., 2006). However, another study from South Africa reported either no or inconsistent association between *p,p′*-DDT and *p,p′*-DDE exposure with oestradiol, testosterone and semen quality, although low concentrations of *p,p′*-DDT and metabolites were observed (Dalvie et al., 2004a,b). Furthermore, exposure to *p,p′*-DDT and metabolites during pregnancy have been speculated to be associated with increased risk of spontaneous abortion, preterm delivery, intrauterine growth restriction, low birthweight, and birth defects whereas other studies in low exposure environments show weak or inconsistent results (reviewed in Rogan and Chen, 2005).

In conclusion, human exposure studies indicate possible toxicological and endocrine-disrupting effects of *p,p′*-DDT and metabolites on human reproduction, however, findings remain inconsistent. Therefore, the use of well controlled *in vitro* bioassay

systems will most likely provide the most plausible toxic effects and mechanistic insights of *p,p′*-DDT exposure.

Table 1.7: Human studies of potential reproductive outcomes of *p,p′*-DDT and *p,p′*-DDE exposure

Study location	Type of study	Number of participants (N)	p,p'-DDT or p,p'-DDE concentration	Outcome	Comments
Mexico[#]	Cross sectional	24 males	*p,p′*-DDE (range: 17.0 – 177.2 µg/g serum lipid)	*p,p′*-DDE level was negatively associated with semen volume, sperm count and free/bound testosterone; positively associated with sex hormone-binding globulin (SHBG)	Low number of subjects
South Africa[#]	Cross sectional	47 male malaria workers	*p,p′*-DDE (range: 1.1–273.5 µg/g serum lipid)	Weak and inconsistent association with oestradiol, testosterone or sperm quality	Study involved occupationally exposed men and this might have affected the outcome
USA[#]	Cross sectional	137 male farmers	*p,p′*-DDE (median: 1.2 µg/g serum lipid or 7.7 µg/L serum)	The tenth percentile of *p,p′*-DDE level was linked to reduced testosterone	
Sweden; Lativa[#]	Cross sectional	110 sea fish eaters	*p,p′*-DDE (median: 0.8 µg/g serum lipid)	*p,p′*-DDE exposure was weakly and negatively associated with testosterone	This association was not significant
Italy[#]	Cross sectional	107 former malaria workers	*p,p′*-DDE (median: 0.4 µg/g serum lipid)	No significant association with oestradiol, testosterone, luteinising hormone, follicle-stimulating hormone, and sex-hormone-binding globulin	Study involved occupationally exposed men and this might have affected the outcome
India[#]	Case control	10 cases and 25 controls	*p,p′*-DDE (mean: 164 µg/L (cases); 13 µg/L serum (controls))	High exposure was associated with higher risk of spontaneous abortion	Low number of participants
China[#]	Case control	15 cases and 15 controls	*p,p′*-DDE (mean: 22 µg/L (cases) and 12 µg/L serum (controls))	Increased exposure was associated with high risk of spontaneous abortion	Low number of participants; Exposure measured after 2 years after abortion which

Study location	Type of study	Number of participants (N)	p,p'-DDT or p,p'-DDE concentration	Outcome	Comments
					may have affected the concentration reported
Italy[#]	Case control	120 cases and 120 controls	*p,p′*-DDE (mean: 5.2 µg/L (cases) and 4.6 µg/L serum (controls))	No significant association between exposure and spontaneous abortion was observed	
USA[#]	Cross sectional	1717 pregnant women	*p,p′*-DDE (median: 25 µg/L serum	Increased exposure with higher frequency of pregnancy loss in prior pregnancies	
Japan[#]	Case control	45 cases and 30 controls	*p,p′*-DDE (mean: 0.7 µg/L (cases) and 0.9 µg/L serum (controls))	No significant association between exposure and spontaneous abortion was observed	
Mexico[#]	Case control	133 cases and 100 controls	*p,p′*-DDE (median: 0.19 µg/g (cases) and 0.15 µg/g serum lipid (controls))	Speculated that increased exposure was associated with increased risk of preterm birth	Noted high confidence intervals and no statistical significance observed
USA[#]	Cohort	2613 pregnant women; 361 preterm and 221 small for gestational age	*p,p′*-DDE (median: 25 µg/L serum	*p,p′*-DDE exposure was strongly associated with higher risk of preterm birth and small for gestational age	Higher association was found between *p,p′*-DDE exposure and preterm birth
USA[#]	Case control	20 preterm cases and 20 full-term controls	*p,p′*-DDE (median: 1.3 µg/L serum (cases) and 1.4 µg/L serum (controls))	No significant association was found between *p,p′*-DDE exposure and preterm birth	Low sample size
India[#]	Case control	15 preterm cases and 25 full-term controls	*p,p′*-DDE (mean: 58 µg/L serum (cases) and 13 µg/L serum (controls))	Higher maternal *p,p′*-DDE level had increased cases of preterm birth	Low sample size
Ukraine[#]	Cross sectional	197 singleton infants	*p,p′*-DDE (median: 2.5 µg/g breastmilk fat	No correlation was found between p,p'-DDE and birthweight after adjusting for	

Study location	Type of study	Number of participants (N)	p,p'-DDT or p,p'-DDE concentration	Outcome	Comments
				potential confounding factors	
India[#]	Case control	30 cases and 24 controls	*p,p′*-DDE (mean: 9 µg/L serum (cases) and 6 µg/L serum (controls))	Observed relationship between maternal blood levels of *p,p′*-DDE and intrauterine growth retardation and birth weight	Study was conducted from areas with high use of *p,p′*-DDT pesticide
USA[#]	Cross sectional	912 infants	Maternal median *p,p′*-DDE at birth: 13 µg/L serum	Maternal serum *p,p′*-DDE was not associated with birthweight	
USA[#]	cohort	119 and 24 frequent and infrequent fish consumers, respectively	*p,p′*-DDE (median: 2 µg/L serum (frequent consumers) and 1 µg/L serum (infrequent consumers))	Log transformed maternal serum p,p'-DDE concentration was inversely associated with birthweight	Number of participants skewed against infrequent fish eaters
Greenland[#]	Cross sectional	178 neonates	Maternal plasma *p,p′*-DDE (mean: 5 µg/L)	No association was found between maternal exposure and neonatal birthweight	
USA[#]	Nested case control	219 cryptorchidism, 199 hypospadias, 167 polythelia, and 552 control babies	*p,p′*-DDE [median: 24 µg/L (cryptorchidism, hypospadias, and controls); 32 µg/L serum (polythelia)]	Serum *p,p′*-DDE level ≥ 60 µg/L tend to show slightly increased birth defects.	Results were inconclusive
USA[#]	Nested case control	75 cryptorchidism, 66 hypospadias, and 283 control	Maternal serum *p,p′*-DDE [median: 43 µg/L (cryptorchidism and control babies) and 41 µg/L	*p,p′*-DDE level ≥ 61 µg/L resulted to slightly increased risk for cryptorchidism and hypospadias, but this was not statistically significant	

Study location	Type of study	Number of participants (N)	p,p'-DDT or p,p'-DDE concentration	Outcome	Comments
		babies	(hypospadias)]		
India[#]	Case control	30 small for gestational age babies and their mothers (cases) and 30 normal birthweight babies and their mothers (controls)	*p,p′*-DDT [maternal blood: 1.5 ± 1.88 ng/L (case) and 1.4 ± 2.02 ng/L (control) ; cord blood: 1.1 ± 1.45 ng/L (case) and 0.9 ± 1.26 (control); Placenta: 1.19 ± 1.60 ng/L (case) and 1.17 ± 1.24 ng/L (control)] .	Significant association between maternal *p,p′*-DDT exposure and low birthweight in neonates	Small sample size
South Africa*****	Cross sectional	535 men	*p,p′*-DDT (range: below detection limit - 519 µg/g lipid) and *p,p′*-DDE (mean: below detection limit - 997 µg/g lipid	Men with high serum *p,p′*-and *p,p′*-DDT concentrations also had high serum testosterone, oestradiol, but low follicle stimulating hormone and luteinising hormone concentrations.	Continuous exposure to *p,p′*-DDE and *p,p′*-DDT may cause alteration of reproductive hormone homeostasis in men
South Africa******	Cross sectional	733 women	*p,p′*-DDT (geometric mean: 67.9 µg/g lipid) and *p,p′*-DDE (geometric mean: 284.2 µg/g lipid)	Maternal exposure to *p,p′*-DDT and *p,p′*-DDE are associated with elevated odds of hypertensive disorders of pregnancy	

Table was prepared from Rogan and Chen, (2005)[#]; Bornman et al., (2018)*, and Murray et al., (2018)**

1.4 Assessment of interactive toxicological effects of chemical mixtures

Humans and animals are continuously exposed to multiple combinations of chemical substances (e.g. personal care products, mycotoxins, food additives and pesticides) through different exposure routes, including inhalation, ingestion and skin contact. However, the current toxicological risk assessment of chemicals for regulatory purposes does not generally take into account the effects of exposure to chemical mixtures, but mainly relies on the assessment of individual substances (Kienzler et al., 2014). In the evaluation of mixture toxicity, the main approaches involve the evaluation of the toxicological effects of individual compounds in the mixture followed by testing the effects induced by the chemical mixtures. The combinatory interactive effects of such chemical mixtures are usually predicted by using different mathematical models (Kienzler et al., 2014). There are several mathematical methodologies which are commonly applied to estimate the combined toxicological effect of known substances and the formulation of the null hypothesis of additivity assumption or non-interaction, including independent addition or response addition and concentration or dose addition (Kienzler et al., 2016). The independent addition model is based on the concept that all components of a mixture affect the same endpoint through different modes of action or target sites (Groten et al., 2001). The independent action (Bliss independence) model is mathematically shown below:

$$E^0_{AB}(d_A,d_B) = E_A(d_A) + E_B(d_B) - E_A(d_A)E_B(d_B) \quad \text{.....(1)}$$

The independent action-expected mixture effect is scaled to the range 0-1 (0-100%) and therefore, $E_A(d_A)E_B(d_B)$ is equivalent to 1 or 100%. In order to evaluate the effects below or above additivity, expected additive values are compared to actually measured values. On the other hand, the concentration addition model assumes that that all compounds in a mixture have an identical mechanism of action (Groten et al., 2001). The concentration additivity (Loewe additivity) model is mathematically shown below:

$$(d_A / D_A) + (d_B / D_B) = I \quad \text{.....(2)}$$

with d_A, d_B denoting combination doses; E_A, E_B, E^0_{AB} representing single-agent and zero interaction combination effects; D_A, D_B denoting doses of agents A and B that produce the same magnitude of effect as a dose combination (d_A,d_B) when used alone; and I representing interaction index.

The application of concentration additivity model requires the determination of isoboles (lines of equal effects) by a systematic variation of combination doses (isobolographic analysis). The shapes of the constructed isoboles in an isobologram provide an indication on the type of interaction. The interaction index (I) near 1 indicates additive effect, I < 1 indicates Loewe synergism, and I > 1 Loewe antagonism of the chemical mixtures.

Based on the above models, additive effect is an effect observed after exposure to two or more chemical agents which act jointly but do not interact. The total effect is the simple sum of the effects of separate exposure to the chemicals under the same conditions. In the other hand, synergism is a toxicological interaction in which the combined observed effect of two or more chemical substances is greater than the predicted expected effect on the basis of the simple summation of the toxicity of each of the individual substances whereas antagonism is when the combined observed effect of two or more chemical mixtures is lower than predicted additive effects (Groten et al., 2001; Kienzler et al., 2014).

In addition, several other prediction models have been applied for studying the interaction of mycotoxin combinations, including central composite design, full factorial design, isobologram analysis/combination index, and the arithmetic definition of additivity (Klarić et al., 2012). The most commonly used is the arithmetic definition of additivity first applied by Weber et al. (2005). The model is based on comparing the calculated expected values obtained from the response of the individual mycotoxins in mixtures with the measured values from combined mycotoxins. The arithmetic definition of additivity is similar to the independent action (Bliss independence) model (Groten et al., 2001). In binary mycotoxin combination exposure, the expected cell viability is calculated as shown below:

Mean viability of binary mixtures (expected in % of substance 1 + substance 2) = mean viability (substance 1 in %) + mean viability (substance 2 in %) - mean cell viability of control (100%).....(3)

The expected standard error of mean (SEM) is calculated as shown below:

Expected SEM (substance 1 + substance 2) = $[(\text{SEM of substance 1})^2 + (\text{SEM of substance 2})^2]^{1/2}$.....(4)

In order to evaluate the effects and interactions of the combined toxins below or above additivity, expected additive values are usually compared to the actual measured values in the co-exposure experiment using an unpaired t-test. Additive effects are reported when measured cell viability values are not significantly above or below the expected values whereas a synergistic or antagonistic interactive effects occur when the measured cell viability values are significantly below or above the expected values, respectively (Weber et al., 2005).

The isobologram/combination index analysis also called the Chou-Talalay method (Chou and Talalay, 1984; Chou, 2006) which was derived from the median-effect principle and the combination index theorem is also used for evaluating the effects of mycotoxin mixtures. This involves the determination of isoboles (lines of equal effects) by a systematic variation of combination doses (isobolographic analysis) to draw a line joining equally effective doses. In the isobologram, the additive effects follow the diagonal line drawn between the effective concentrations of each single compound in the mixtures. If the measured combined effect of two chemicals is below or above the diagonal line, it indicates synergism and antagonism, respectively. A combination index is also applied in identifying the interactive effect between two or more mycotoxin mixtures (Chou, 2006). Similar to the Loewe interaction index, a combination index value of 1 indicates additive effects whereas combination index less than 1 indicates synergism and combination index value greater than 1 indicates antagonism. The major drawback of the combination index-Isobolgram method as described by Chou (2006) is that it cannot be applied when data sets exceeded 100% and values greater than 100% growth cannot be entered into CalcuSyn (Biosoft®, Cambridge, UK) or CompuSyn (ComboSyn Inc., USA) softwares, making the calculation of the isobologram-combination index not applicable.

1.5 Aims and objectives of the study

1.5.1 Aims of the study

The overall aim of this thesis is to evaluate the potential of individual mycotoxins and persistent organochloride pesticides, and mixtures of these, for causing reproductive and developmental toxicity by assessing response of reproductive cells after exposure to biologically and environmentally relevant concentrations.

1.5.2 Specific objectives

The following are the specific objectives of the study:

1. To assess the effect of selected mycotoxins and persistent organochloride pesticides on oestrogen receptor (ER) transcriptional response using MMV-Luc reporter gene assay.
2. To evaluate the testicular toxicity of mycotoxins and persistent organochloride pesticides, either individually or as mixtures, *in vitro* using MA-10 murine Leydig cell line.
3. To evaluate the effects of mycotoxins and persistent organochloride pesticides, either individually or as mixtures, on the viability, endocrine and metabolic function of the human placenta using BeWo cell line as an *in vitro* model.

Chapter 2

2. General materials and methods

The laboratory experiments of the results reported in this thesis were performed at the Institute for Global Food Security, School of Biological Sciences, Queen's University Belfast, United Kingdom as well as at the School of Food Science and Nutrition (FSN), and Leeds Institute of Cardiocvascular and Metabolic Medicine (LICAMM) at the School of Medicine, both in the University of Leeds, United Kingdom.

2.1 Chemicals, disposables and cell lines

Aflatoxin B_1 (AFB_1; purity ≥ 98%), deoxynivalenol (DON; purity ≥ 98%), zearalenone (ZEN; purity ≥ 98%), ochratoxin A (OTA; purity ≥ 98%), alpha-zearalenol (α-ZOL; purity ≥ 98%) and beta-zearalenol (β-ZOL; purity ≥ 98%), 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane (*p,p'*-DDT; purity ≥ 98%), and 1,1-dichloro-2,2-bis(p-chlorophenyl) ethylene (*p,p'*-DDE; purity ≥ 98%), absolute ethanol, dimethyl sulfoxide (99.99%), 17β-estradiol (E_2; purity ≥ 98%), fulvestrant (ICI 182,780; purity ≥ 98%), progesterone (P_4; purity ≥ 99%), general foetal bovine serum, charcoal-stripped foetal bovine serum, Trypan blue, 3-[4,5-dimethylthiazol-2-yl]-2,5-dipenyltetrazolium bromide solution (MTT; 5mg/mL in PBS), solubilising reagent [10% sodium dodecyl sulfate (SDS) and 0.01 M hydrochloric acid (HCl)], Chemgene and ELISA plate sealers were purchased from Sigma-Aldrich (Dorset, UK). The MA-10 Leydig cell line (ATCC® CRL-3050™), Dulbecco's phosphate buffered saline (PBS) and 0.1% Gelatin solution (sterile) were purchased from LGC Standards (Middlesex, UK). Dulbeco's Modified Eagle Medium/F-12 nutrient mixture (DMEM/F-12) without phenol red, Dulbeco's Modified Eagle Medium (DMEM) without phenol red, DMEM/F-12 GlutaMax supplement with phenol red, HEPES (1M), RNase/DNase free water, TrypLE™ Express, Countess™ cell counting chamber slides, penicillin-streptomycin and 0.4% trypan blue were obtained from Invitrogen™ Life Technologies (Paisley, UK). The 96 well microtitre plates for MTT assay were obtained from Nunc, Rosklide, Denmark, the white-walled 96-well plates with clear flat bottoms used for reporter gene assay (RGA) were from Greiner Bio-One, Frickenhausen, Germany whereas the cell culture flasks, 24 well, 12 well, and 6 well plates were from Corning Incorporated, New York. The PCR plates, PCR plate sealers and RNase/DNase/pyrogen free pipette tips

were purchased from StarLab, UK. The Cayman's oestradiol and testosterone ELISA kits (Cayman Chemical, Ann Abor, USA) were purchased from Bertin Pharma (Montigny-le-Bretonneux, France) while progesterone and beta-human chorionic gonadotrophin ELISA kits were from DRG Diagnostics, Germany. Applied Biosystems High Capacity cDNA Reverse Transcription Kit purchased from Thermo Fisher Scientific, RNeasy Mini Kit from Qiagen Ltd, All-in-One^{TM} qPCR mix and Rox dye were from GeneCopoeia, USA while the gene-specific primers were purchased from 2BScientific, UK. The BeWo cell line (ECACC 86082803) was a kind gift from Dr. Karen Forbes of Manchester University, UK whereas the MMV-Luc cell line was obtained from Dr. Lisa Connolly of the School of Biological Science at Queen's University Belfast, UK. All other chemicals are of analytical grade and used without further purification or treatment. AFB_1, DON, ZEN, α-ZOL, β-ZOL, OTA, *p,p'*-DDT and *p,p'*-DDE were dissolved in DMSO (99.99%) to make stock solutions and working solutions were prepared in assay media to give a DMSO concentration of 0.1% (v/v). The final concentration of DMSO (0.1% v/v single toxin treatment and 0.2% v/v in binary toxins treatment) equivalent to the highest DMSO concentration of working dilutions was tested and results were not significantly different from untreated media controls. The molar and gramme concentration of the mycotoxins and persistent organochloride pesticides used in this study is shown in **Appendix 1**. The doses of mycotoxins and pesticides used were based on concentrations reported in human and animal exposure studies (**Table 2.1**) as well as in previous *in vitro* studies (**Table 2.2**).

Table 2.1: Exposure levels of mycotoxins and persistent organochloride pesticides

Compound	Doses (ng/mL)	Doses (µM)	Reference
Serum OTA	18.4 - 171.3	0.046 - 0.424	Hassen et al., 2004
Serum OTA	23.0 - 66.2	0.057 - 0.164	Abid et al., 2003
Serum OTA	1800	4.458	Steyn, 1993
Serum OTA	1136	2.813	Maaroufi et al., 1995
Urinary DON	436	1.471	Sarkanj et al., 2013
Urinary DON	1238	4.178	Wells et al., 2016
Serum ZEN	200 – 475	0.628 - 1.492	Pillay et al., 2002
Serum α –ZOL	70 – 707	0.218 - 2.207	Pillay et al., 2002
Serum β-ZOL	110 – 707	0.343 - 2.207	Pillay et al., 2002
Serum ZEN	167.0±17.1	0.525±0.054	Tomaszewski et al., 1998
α –ZOL from Bile (Cattle)	9.9 – 489	0.031 - 1.526	Kennedy et al., 1998
β-ZOL from Bile (Cattle)	21.3 - 1,244.5	0.066 - 3.884	Kennedy et al., 1998
Urinary ZEN	2690 - 6870	8.450 – 21.579	Mirocha et al., 1981
Urinary α –ZOL	2970 – 6000	9.270 – 18.728	Mirocha et al., 1981
Urinary β-ZOL	1970 – 2660	6.149 – 8.303	Mirocha et al., 1981
Urinary ZEN (pig)	2300 - 36000	7.23 - 113.10	Mirocha et al., 1981
Urinary α –ZOL (pig)	800 - 3800	2.50 - 11.87	Mirocha et al., 1981
Urinary β-ZOL (pig)	600 - 700	1.87 - 2.17	Mirocha et al., 1981
Urinary ZEN (rat)	5000 - 47500	15.71 - 149.20	Mirocha et al., 1981
Urinary α –ZOL (rat)	900 - 4100	2.81 - 12.80	Mirocha et al., 1981
Urinary β-ZOL (rat)	900 - 1250	2.81 - 3.90	Mirocha et al., 1981
Serum *p,p'*-DDT	390 - 14290	1.1 - 40.311	Dua et al., 2001
Serum *p,p'*-DDE	100 - 4650	0.314 - 14.621	Dua et al., 2001

Compound	Doses (ng/mL)	Doses (µM)	Reference
Serum *p,p'*-DDE	27400	66.16	Longnecker et al., 2002
Serum AFB_1	1392	4.458	Uriah et al., 2001
AFB_1 (semen)	1,660±40	5.316±0.13	Ibe et al., 1994
Serum AFB_1	8045	25.763	Onyemelukwe et al., 2012

Table 2.2: Concentrations of mycotoxins used in previous *in vitro* studies

Compound	Doses (µM)	Cell line	Reference
ZEN	6.25 – 25	CHO-K1	Tatay et al.., 2016
α –ZOL	6.25 – 25	CHO-K1	Tatay et al.., 2016
β-ZOL	6.25 – 25	CHO-K1	Tatay et al.., 2016
ZEN	10 - 60	HCT116	Bensassi et al., 2014
DON	20 - 200	HCT116	Bensassi et al., 2014
DON	10 - 100	J774A.1	Marzocco et al., 2009
DON	4 - 20	Caco-2	Kouadio et al., 2007
ZEN	10 - 40	IPEC-J2	Wan et al., 2013
ZEN	12.5 - 100	CHO-K1	Tatay et al.., 2014a
α –ZOL	6.5 - 100	CHO-K1	Tatay et al.., 2014a
β-ZOL	6.5 - 75	CHO-K1	Tatay et al.., 2014a
OTA	10 - 50	Vero	Ramyaa et al., 2013
OTA	0.25 - 50	HepG2	Bösch-Saadatmandi et al., 2006
OTA	10 - 50	hPMBC	Periasamy et al., 2016
ZEN	0.5 - 50	HepG2	Wang et al., 2014c
α –ZOL	1 - 100	HepG2	Wang et al., 2014c
ZEN	1 - 100	Caco-2	Vejdovszky et al., 2016
DON	0.01 - 10	Caco-2	Vejdovszky et al., 2016
AFB_1	1 - 70	PK-15	Lei et al., 2013

2.2 Laboratory equipment

The list of laboratory equipment used in this research work are as follows:

Countess® automated cell counter (Invitrogen Life Technologies), Eppendorf 5415C microcentrifuge and Eppendorf centrifuge 5810R (Palo Alto, Califonia, USA), Millipore Direct-Q5 water purification system (Millipore, Watford, UK), Multiskan FC® Plate reader (Thermo Scientific, Vantaa, Finland), iEMS microplate reader (Thermo Scientific, Langenselbold, Germany), ELISA plate shaker (Thermo Scientific, Vantaa, Finland), D-79219 Model Magnetic stirrer (IKA-Werke, Staufen, Germany), ABT PRISM 7900HT Sequence detection system and Veriti 96 Well Thermal Cycler (Applied Biosystems, UK), Olympus inverted microscope, Water bath, Freezers (-20°C and -80°C), refrigerator, class II biological laminar flow cabinet, weighing balance, Mithras LB 940 Multimode luminometer (Berthold, Other, Germany), Cryostat and cell culture incubator

2.3 General Cell culture

All the cell culture procedures were performed inside a class II biological laminar airflow cabinet. Strict aseptic technique was followed in all the cell culture and media preparation procedures. Before use, the laminar flow cabinet was switched on and monitored for its safety of use for at least 15 minutes. Thereafter, it was cleaned with 2% Chemgene followed by 70% ethanol before and after each use. All items brought into the flow cabinet were also swabbed with 70% ethanol. At each cell culture work, only one cell line was used inside the laminar flow cabinet and the cabinet was allowed to clear for at least 15 minutes before another activity is taken inside it. Each cell line was assigned a specific bottle of culture media. This was to eliminate any possibility of cross contamination between various cell lines. In addition, all pippete tips, universal bottles, Eppendorf tubes, and stripipettes used were sterile.

Sterility tests were also routinely performed on all cell culture reagents directly after preparation and prior to use. After the preparation of a new media, 10 mL was aseptically transferred to T25 cell culture flask and incubated for 3 days at 37 °C. The

flask was observed daily for media colour change and microscopically for the presence of microbial contamination.

2.3.1 MA-10 Leydig cell culture

The MA-10 Leydig cell line (ATCC® CRL-3050™) was routinely cultured in DMEM/F-12 GlutaMax supplement (with phenol red) containing 15% charcoal-stripped foetal bovine serum, 2% HEPES, and 1% penicillin-streptomycin. Cells were maintained in a 75 cm^2 cell culture flask (Corning, Corning Incorporated, New York) at 37 °C in a humidified atmosphere (95%) with 5% CO_2. The flasks were pre-coated at room temperature with sterile 0.1% gelatin solution. The cells were passaged every 3 days.

2.3.2 BeWo Placental cell culture

The BeWo cell line (ECACC 86082803) was routinely cultured in DMEM/F-12 GlutaMax supplement (with phenol red) containing 10% foetal bovine serum and 1% penicillin-streptomycin. Cells were maintained in a 75 cm^2 cell culture flask (Corning, Corning Incorporated, New York) at 37 °C in a humidified atmosphere (95%) with 5% CO_2 and cells were splited every 3 - 4 days.

2.3.3 MMV-Luc cell culture and Reporter Gene Assay

The MMV-Luc cell line specific for the detection of oestrogen receptor transcriptional activation was previously developed from the MCF-7 cells (ATCC HTB-22) by transformation with luciferase reporter construct under an oestrogen inducible promoter (Willemsen et al., 2004). MMV-Luc cells were cultured in DMEM (without phenol red) containing 10% charcoal-stripped foetal bovine serum without antibiotics and maintained in a 75 cm^2 cell culture flask (Nunc, Roskilde, Denmark) at 37 °C in a humidified atmosphere (95%) with 5% CO_2 for at least 48 h before use to ensure that the cells are completely free from hormonal induction from the media.

For experiments, attached cells were liberated from flasks using TrypLE™ Express trypsin (Invitrogen™ Life Technologies, Paisley, UK), counted for viability checks

prior to seeding plates by trypan blue staining and using a Countess® automated cell counter (Invitrogen™ Life Technologies, Paisley, UK). Cells (100 µL) were seeded at a density of 4 x 10^5 cells/mL into each well of white walled 96-well plates with clear flat bottoms (Greiner Bio-One, Frickenhausen, Germany) and incubated for 24 hours to allow cells to attach before chemical treatment. The cells were then exposed to different concentrations of mycotoxins, reference hormones and fulvestrant (ICI 182,780; ER antagonist) diluted with media to the final concentrations: ZEN (0.01 – 1000 nM), α-ZOL (0.005 – 50 nM), DON (1 – 30,000 nM), OTA (1 – 30,000 nM), E_2 (0.0005 – 10 nM), fulvestrant (ICI 182,780; 1 µM), progesterone (500 nM), and methanol (0.5%) and media controls. The MMV-Luc reporter gene assay was performed as previously reported (Frizzell et al., 2011; Ndossi et al., 2012), except that cells were treated with chemicals diluted with media without antibiotics and were incubated for 48 h instead of 24 h. The presence of antibiotics in media causes high background luciferase response (Wilson et al., 2004). It has been shown that the optimum level of hormone production and gene expression occur after 48 h in H295R adrenocortical cell line (Rainey et al., 1993; Gracia et al., 2006; Hecker et al., 2006), JEG-3 placental cell line (Woo et al., 2013), and BLTK1 Leydig cell line (Forgacs et al., 2012). Therefore, 48 h was chosen for the reporter gene assay and other assays to allow for direct comparison between parameters. After incubation for 48 hours, the media supernatants were discarded, the cells washed once with sterile PBS and lysed with 25 µL of lysis buffer (Promega, Southampton, UK; Cat. No.: E1531). Finally, 100 µL of luciferase enzyme (Promega, Southampton, UK; Cat. No.: E1501) was injected into each well and the luciferase activity in relative light units (RLUs) were measured using the Mithras LB 940 Multimode luminometer (Berthold, Other, Germany). The luminescence readings of each treatment was normalised to the readings of E2 alone (10 nM) and this was taken as the maximum response (100%). Vehicle (0.5% methanol) controls were used to define the minimum response (0%). Measurements of 10 nM E_2, 0.5% methanol and progesterone were conducted in parallel on every single plate and served as positive, solvent and negative controls, respectively.

The mycotoxins and pesticides with relative estrogenic response above 10% were selected and tested together with E_2 (0.05 nM and 10 nM) either in single or binary combinations to ascertain their additive and antagonistic and effects on E_2–mediated ER

transcriptional response. The 0.05 nM and 10 nM of E_2 were chosen as they fall within the reference ranges of serum concentrations of E_2 seen in females in pre-pubertal stage, during puberty and in late pregnancy (JECFA; 2000; Elmlinger et al., 2002). The normal serum E_2 concentration is usually in the range 8 – 18 pg/mL (0.029 – 0.066 nM) in prepubertal women, 20 – 350 pg/mL (0.073 – 0.35 nM) in premenopausal women and this reaches peak level (18,000 pg/mL: 66.1 nM) in late pregnancy (JECFA; 2000; Elmlinger et al., 2002). Furthermore, circulating E_2 level ranges ≤ 10 – 50 pg/mL (0.037 – 0.184 nM) both in prepubertal and adult men, depending on age (JECFA; 2000).

The concentrations of ZEN (0.01 nM - 1000 nM), α-ZOL (0.005 nM - 50 nM), *p,p'*-DDT (1 μM - 50 μM) and *p,p'*-DDE (1 μM - 50 μM) were used in single or binary combinations tested together with the selected concentrations of E_2. After exposure of the cells to the mycotoxins and pesticides together with E_2 (0.05 nM and 10 nM) either in single or binary combinations for 48 hours, the media supernatants were discarded, the cells washed once with PBS and lysed with 25 μL of lysis buffer (Promega, Southampton, UK). Finally, 100 μL of luciferase enzyme (Promega, Southampton, UK) was injected into each well and the luciferase activity in relative light units (RLU's) were measured using the Mithras Multimode Reader (Berthold, Other, Germany). The luminescence readings of each combined treatment was normalised to the readings of E2 alone (10 nM) and this was taken as the maximum response (100%). Vehicle (0.5% methanol) controls were used to define the minimum response (0%).Measurements of E_2 (0.05 nM and 10 nM), 0.5% methanol and progesterone were conducted in parallel on every single plate and served as positive, solvent and negative controls, respectively. The high affinity ER antagonist ICI 182,780 was used to verify whether the detected estrogenic responses are ER dependent. E_2 (0.05 nM and 10 nM) was co-incubated with 1 μM of ICI 182,780 and the inhibition of oestrogenic response of E_2 confirmed that the responses observed in the assay were mediated oestrogen receptors (ERs). Progesterone was tested in each plate as a negative control and it had no estrogenic response.

2.3.4 Passaging and counting cells

When the cells reached 80 - 90% confluent in the T75 flask, they were prepared for passaging. All the old media was removed completely from the flask. The cells were

washed once with 5 ml of sterile PBS. Then 3 ml of Trypsin-EDTA (10 fold diluted) was added to the T75 flask which was enough to cover the whole bottom surface of the flask. The trypsinised cells were incubated for 3 to 5 minutes. Afterwards, all cells were detached from the bottom surface of the flask and 7 ml of pre-warmed assay media was added to flask to stop trypsinisation. Cells were aseptically transferred into a sterile 15 ml tube and centrifuged for 5 min at 1200 RPM. Then, the media supernatant was discarded and the cell pellet was re-suspended with 1 ml of assay media with respect to the cell line.

After the cells were re-suspended with 1 ml fresh assay media, 4 µl of cell suspension was transferred into a 0.5 mL eppendorf containing 36 µl of Trypan Blue and the content was mixed gently by pipetting up and down. The improved Neubauer counting chamber (Hawksley, Sussex, UK) was prepared for counting cells by placing cover slip onto the grooves on the glass slide. Then 10 µl of mixed cell suspension was transferred to one side of the slide under the cover slip and each groove was filled by capillary action. Thereafter, cells were counted in the large square in four corners (A parts: each containing 16 small squares) of the slide (**Fig. 2.1**).

For the Countess® automated cell counter method, 10 µL of 0.4% Trypan blue (Invitrogen Life Technologies, Paisley, UK. Cat. No.: T10282) and applied to Countess® cell counting chamber slides (Invitrogen Life Technologies, Paisley, UK. Cat. No.: C10283) before inserting the counting slide into the counting chamber. Then the total number of viable and non-viable cells per mililitre were calculated automatically. Based on the experiment requirements, the counted cells were diluted and different number of cells were seeded to either flasks or plates.

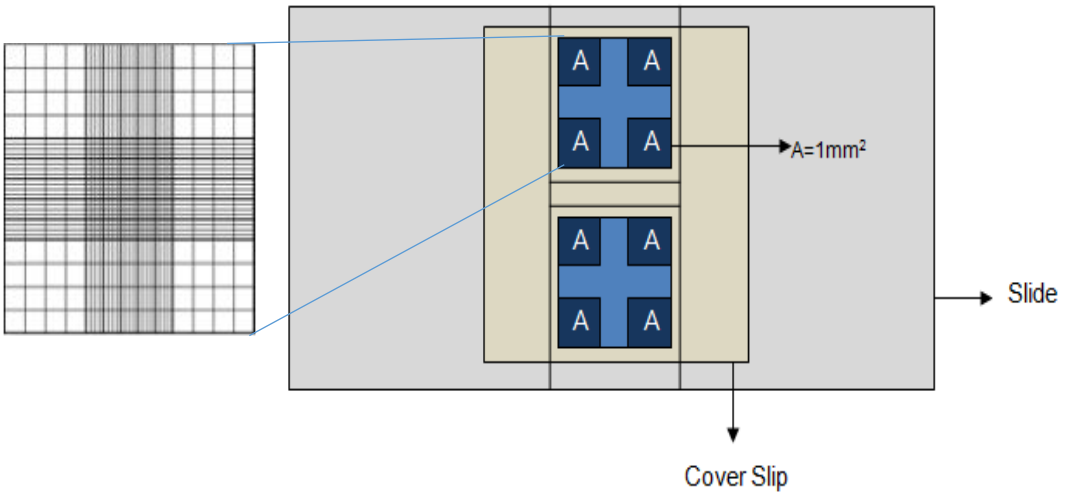

Fig. 2.1: Improved Neubauer chamber**.** The cells were counted in the area A which have 16 small squares each. For each light blue square, the number of cells in the four A parts were added up to obtain the number of cells counted for each well. The two wells (eight A squares) were counted and the average taken to obtain the total number of cells. Then the number of cells per ml of cell suspension was calculated following the formula: [number of cells counted x dilution factor (usually 10) x $10^4 \div 4$].

2.3.5 Freezing cells (Cryopreservation)

Cells from various cell lines of different passages were frozen and cryo-preserved in liquid nitrogen as master stocks that can be thawed and used for further research if necessary. The freezing media was prepared with 90% fetal bovine serum and 10% of sterile DMSO (Sigma-Aldrich, Poole, Dorset, UK; Cat. No.: D2650). After counting the cells as shown in section 2.3.4, cells were pelleted and resuspended in routine culture media for MA-10 cells or foetal bovine serum for BeWo cells which were pre-cooled in 4°C. 500 µl of cell suspension was transferred to a cryovial (Nalgene, Roskilde, Denmark; Cat. No.: 5000-1020) previously labelled with the name of cell line, date, passage number and name of research group. Then equal volume (500 µl) of the freezing media which has been pre-cooled in 4°C was added to the cryovial containing the cell suspension in dropwise. The cell number would be at least 1.0 x 10^6 cells per ml. The vials were placed into a Nalgene Mr Frosty container (Sigma-Aldrich, Poole, Dorset, UK; Cat. No.: 34863) which is a polycarbonate container holding isopropanol and was kept at -20°C freezer prior to use. Isopropanol controls the cooling at a constant

rate of 1°C per minute which was required for successful cryopreservation of cells. The Mr Frosty container was then immediately placed in the -80°C freezer overnight. Thereafter, the vials were moved to the liquid nitrogen storage tank for long term storage.

2.3.6 Cell thawing (Breaking out cells)

The vials containing cryopreserved cells were removed from the liquid nitrogen tank and thawed quickly by placing the vial in a 37 °C water bath ensuring the cap was above the water level. When the cells were thawed, the vial was removed from the water bath, cleaned with 70% ethanol and the cell suspension was rapidly transferred to a sterile 15 mL universal tube containing 9 mL of fresh culture media inside a laminar flow cabinet. The cryopreserved cells were quickly transferred to fresh media after thawing to reduce their exposure time to DMSO as DMSO at > 0.5% is toxic to cells at room temperature. Then the tube was centrifuged at 1200 RPM for 5 minutes at room temperature. Thereafter, the tube was removed, media supernatatnt decanted, and cell pellet re-suspended in 1 mL of fresh culture media. The cells were seeded into T25 (25 cm^2) tissue culture flask and allowed to attach overnight. The following day, the old media was discarded and replaced with fresh media to remove any residual DMSO from the freezing media. The cells were observed daily and when growth reached 80 – 90% confluence, they were transferred into T75 (75 cm^2) cell culture flasks.

2.4 MTT Cytotoxicity assay

The cytotoxic effects of mycotoxins and persistent organochloride pesticides was measured by using the 3-[4,5-dimethylthiazol-2-yl]-2,5-dipenyltetrazolium bromide (MTT) reduction assay. The MTT assay have been used widely in *in vitro* toxicological studies to measure cellular proliferation and cytotoxicity of chemical exposure, including mycotoxins (Alassane-Kpembi et al. 2015; Clarke et al. 2014) and DDT (Strong et al. 2015). The principle of MTT assay is that yellow tetrazollium salt (MTT) can be converted to a photometrically measurable purple formazan derivative by mitochondrial enzyme succinate dehydrogenase of viable cells. The MTT reagent is absorbed by live cells and passed into the mitochondria to form an insoluble, dark purple formazan product (**Fig. 2.2**). After the addition of solubilising solution (an

organic solvent), the dark purple formazan product becomes solubilised and released. The higher the mitochondria activities in the cells, the darker colour formed and and this is directly proportional to the number of viable cells. The solubilised formazan product was measured spectrophotometrically (Mosmann 1983).

There are several methods that have been employed in evaluating the cytotoxic effects of environmental and food toxicants. MTT, NR (Neutral red), lactate dehydrogenase (LDH), high content assay and AlamarBlue^{TM} assays have been applied to assess the cytotoxicity of different environmental and food toxins on various cells (Clarke et al. 2014; Frizzell et al. 2011; Lei et al. 2013; Strong et al. 2015). It is interesting to note that MTT results have been well correlated with results obtained using neutral red assay (Alassane-Kpembi et al. 2013; Clarke et al. 2014), AlamarBlue^{TM} assay (Ndossi et al. 2012) and the data from high content assay, especially the cell number, nuclear intensity, nuclear area and mitochondria membrane potential (Wilson et al. 2016). Therefore, MTT continues to be a useful method for the indirect assessment of cell proliferation and viability. In this present study, the MTT assay was used to elucidate the cytotoxicity of the selected mycotoxins and pesticides in single and binary combinations to ascertain the cellular mitochondria function of MA-10 Leydig cell line and BeWo placental cell line after exposure for 48 hours.

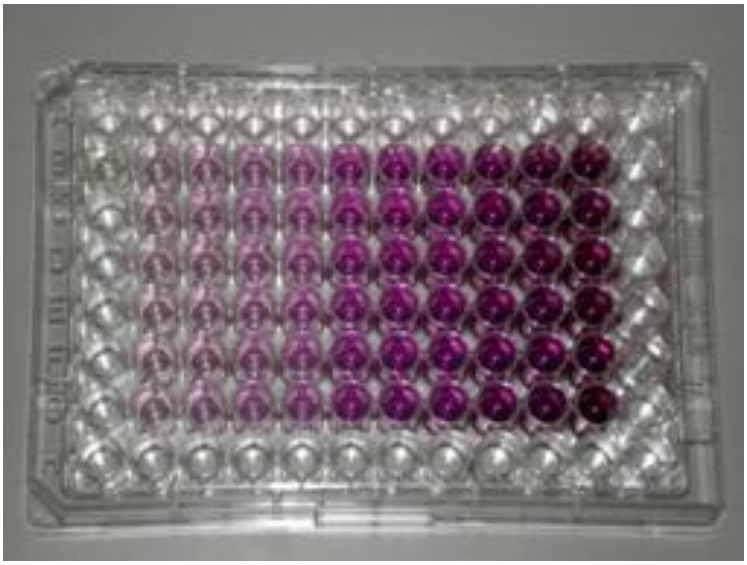

Fig. 2.2: The 96-well plate showing MTT cytotoxicity assay. Viable cells absorb the MTT reagent and this is passed into the mitochondria to form an insoluble, dark purple

formazan product which is dissolved by the addition of the solublising reagent. The intensity of the purple colour is directly proportional to the number of viable cells. The plate is read then read using a micotitre plate reader.

2.4.1 MMV-Luc Cell viability and cytotoxicity assay

The MMV-Luc cells were seeded and treated as described for the reporter gene assay (RGA) in section 2.3.3. After 48 hours of treatment, 20 µL of MTT labeling reagent (5mg/mL) was added into each well containing cells growing in 200 µL of medium and plates covered with aluminum foil. The plates were incubated for 4 hours at 37 °C in a humidified atmosphere (95%) with 5% $CO_{2.}$ Thereafter, 100 µL of solubilisation solution (10% SDS in 0.01 M HCl) was added to each well and incubated overnight at 37 °C in a humidified atmosphere (95%) with 5% $CO_{2.}$ The plates were then read at 570 nm with a reference wavelength of 690 nm with TECAN Safire2 (TECAN, Switzerland) microtitre plate reader. Viability of the each test sample was calculated as the percentage (%) absorbance when compared with the absorbance of the 0.5% methanol vehicle control.

2.4.2 Treatment of MA-10 cells for cytotoxicity assay

MA-10 cells at 80% confluence were detached from flasks using TrypLE™ Express trypsin. The cells were counted for viability by trypan blue staining using an improved Neubauer counting chamber (Hawksley, Sussex, UK). Then cells were seeded into the sterile 0.1% gelatin-coated 96-well culture plates (Nunc, Roskilde, Denmark) at a density of 2 x 10^4 cells per well in 200 µL, 2 x 10^5 cells per well in 200 µL or 3 x 10^4 cells per well in 200 µL cultural media and allowed to attach for 24 hours. This was followed by observing each well for confluence and wells treatd with MTT reagent to determine the optical density of each well. This optimisation experiment was done to ensure that cells are not at 100% confluence before toxin treatment. The 3 x 10^4 cells per well in 200 µL showed 70 - 80% confluence after 24 hours with the best optical density (**Fig. 2.3**) and this cell number was selected for subsequent experiments.

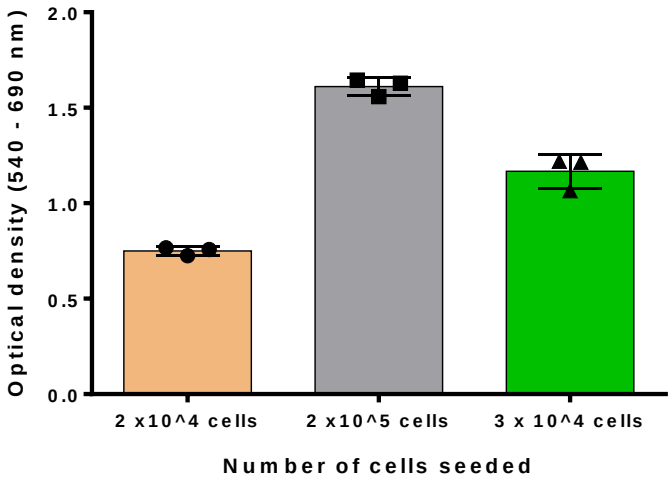

Fig. 2.3: The optical density of different number of MA-10 cells after 24 hours incubation. Data presented are results of three independent experiments with three technical replicates and error bars represents standard deviation of three biological replicates.

The MA-10 cells were seeded into the sterile 0.1% gelatin-coated 96-well culture plates (Nunc, Roskilde, Denmark) at a density of 3 x 10^4 cells per well in 200 µL cultural media and allowed to attach for 24 hours before treatment with mycotoxins and pesticides. Final concentrations of mycotoxins and pesticides were achieved by adding DMEM/F12 media giving a final DMSO concentration of 0.1% (v/v). Media used to dilute mycotoxins and pesticides were free from phenol red and contained only 5% charcoal-stripped foetal bovine serum in order to avoid hormonal stimulation of cell proliferation which may be induced by phenol and general foetal bovine serum. Cells were treated with individual mycotoxins concentrations from 0.1 µM to 64 µM; AFB_1, DON, ZEN, α-ZOL, β-ZOL and OTA, and pesticides (0.1 - 64 µM); *p,p′*-DDT and *p,p′*-DDE and incubated at 37 °C in a humidified atmosphere (95%) with 5% CO_2. Binary mixtures (*p,p′*-DDT/*p,p′*-DDE, *p,p′*-DDT/AFB_1, *p,p′*-DDT/DON, *p,p′*-DDT/OTA, *p,p′*-DDT/ZEN, *p,p′*-DDT/α-ZOL, *p,p′*-DDT/β-ZOL, *p,p′*-DDE/AFB_1, *p,p′*-DDE/DON, *p,p′*-DDE/OTA, *p,p′*-DDE/ZEN, *p,p′*-DDE/α-ZOL, *p,p′*-DDE/β-ZOL, AFB_1/DON, AFB_1/OTA, AFB_1/ZEN, AFB_1/α-ZOL, AFB_1/β-ZOL, DON/OTA, DON/ZEN, DON/α-ZOL, DON/β-ZOL, OTA/ZEN, OTA/α-ZOL, OTA/β-ZOL, ZEN/α-ZOL, ZEN/β-ZOL, and α-ZOL/β-ZOL) with concentrations varying from 0.1 µM to 64 µM were also prepared and both the individual and binary mixtures were tested on the cell line for 48

h. DMSO (0.1% and 0.2% v/v) was used as a vehicle controls for single toxin treatment and binary toxin treatment, respectively.

2.4.3 Treatment of BeWo cells for cytotoxicity assay

BeWo cells at ~ 80% confluence were detached from flasks using TrypLE™ Express trypsin. The cells were counted for viability by trypan blue staining using an improved Neubauer counting chamber (Hawksley, Sussex, UK) and diluted with phenol-free DMEM/F12 media supplemented with 10% charcoal-stripped foetal bovine serum and 1% penicillin-streptomycin. Before the comencement of toxin treatment, BeWo cells at a density of 3 x 10^4 cells per 200 µL of culture media were seeded and incubated for 24 hours after which cells were treated with MTT reagent and optical density measured at 540 nm with a reference wavelength of 690 nm using an iEMS microplate reader (Thermo Scientific, Langenselbold, Germany). It was observed that the optical density at 24 hours was below that obtained for the same cell density of MA-10 cells after 24 hours (**Fig. 2.3 & Fig. 2.4**). Therefore, , BeWo cells at a density of 3 x 10^4 cells per 200 µL of culture media were seeded and incubated for 48 hours after which cells were treated with MTT reagent and optical density measured. The optical density of BeWo cells seeded at 3 x 10^4 cells were similar to those obtained for MA-10 cells at 24 hours (**Fig. 2.4**) and then 48 hours was chosen for subsequent experiments.

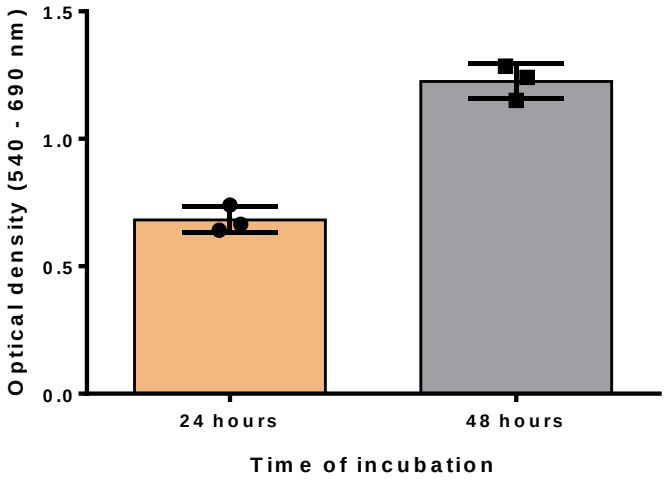

Fig. 2.4: The optical density of BeWo cells seeded at 3 x 10^4 cells per 200 µL incubated for either 24 or 48 hours. Data presented are results of three independent experiments

with three technical replicates each and error bars represents standard deviation of three biological replicates.

BeWo cells were seeded into each well of the 96-well culture plates (Nunc, Roskilde, Denmark) at a density of 3 x 10^4 cells per well in 200 µL cultural media and incubated for 48 hours before treatment with mycotoxins and pesticides. Final concentrations of mycotoxins and pesticides were achieved by adding DMEM/F12 media giving a final DMSO concentration of 0.1% (v/v). DMEM/F12 media used to dilute mycotoxins and pesticides were free from phenol red and contained only 5% charcoal-stripped foetal bovine serum and 1% penicillin-streptomycin in order to avoid hormonal/oestrogenic stimulation of cell proliferation which may be induced by phenol and general foetal bovine serum. Cells expressing ERs such as MCF-7 cells are unable to proliferate when grown in media without oestrogens whereas its proliferation was induced by the addition of oestrogens in culture media (Schirilo et al., 2012). Cells were treated with individual mycotoxins concentrations from 0.1 µM to 64 µM; AFB_1, DON, ZEN, α-ZOL, β-ZOL and OTA, and pesticides (0.1 - 64 µM); *p,p′*-DDT and *p,p′*-DDE and incubated at 37 °C in a humidified atmosphere (95%) with 5% CO_2. Equimicromolar binary mixtures (*p,p′*-DDT/*p,p′*-DDE, *p,p′*-DDT/AFB_1, *p,p′*-DDT/DON, *p,p′*-DDT/OTA, *p,p′*-DDT/ZEN, *p,p′*-DDT/α-ZOL, *p,p′*-DDT/β-ZOL, *p,p′*-DDE/AFB_1, *p,p′*-DDE/DON, *p,p′*-DDE/OTA, *p,p′*-DDE/ZEN, *p,p′*-DDE/α-ZOL, *p,p′*-DDE/β-ZOL, AFB_1/DON, AFB_1/OTA, AFB_1/ZEN, AFB_1/α-ZOL, AFB_1/β-ZOL, DON/OTA, DON/ZEN, DON/α-ZOL, DON/β-ZOL, OTA/ZEN, OTA/α-ZOL, OTA/β-ZOL, ZEN/α-ZOL, ZEN/β-ZOL, and α-ZOL/β-ZOL) with concentrations varying from 0.1 µM to 64 µM were also prepared and both the individual and binary mixtures were tested on the cell line for 48 hours. DMSO (0.1% and 0.2% v/v) was used as a vehicle controls for single toxin treatment and binary toxins treatment, respectively.

2.4.4 MTT Cytotoxicity assay for MA-10 and BeWo cell lines

After the chemical treatment of either MA-10 or BeWo cells for 48 hours, 10 µL of MTT solution was added into each well and plates covered with aluminum foil to reduce exposure to light. The plates were incubated for 4 hours at 37 °C in a humidified atmosphere (95%) with 5% CO_2. Thereafter, 100 µL of solubilising reagent (10% SDS and 0.01 M HCl) was added to each well and incubated overnight at 37 °C in a

humidified atmosphere (95%) with 5% CO_2. The plates were then read at 540 nm with a reference wavelength of 690 nm using an iEMS microplate reader (Thermo Scientific, Langenselbold, Germany). Viability of each sample was calculated as the percentage (%) absorbance when compared with the absorbance of the 0.1% DMSO (single treatment) or 0.2% DMSO (binary treatment) vehicle control. There was no significant statistical difference between the viability of cells treated with 0.1% DMSO compared to those grown in either 0.2% DMSO or media.

2.5 Hormone quantification

Enzyme-linked immunosorbent assay (ELISA) techniques were used to measure the concentration of progesterone (P4), testosterone, oestradiol (E2) and beta-human chorionic gonadotropin (β-hCG) in cell media supernatant, depending on the cell line.

2.5.1 Treatment of MA-10 Leydig cells for hormone assay

Before the commencement of the experiment, MA-10 cells were grown in T75 flasks with DMEM/F-12 media without phenol red and containing 15% charcoal-stripped foetal bovine serum, 2% HEPES, and 1% penicillin-streptomycin for at least 24 hours to starve the cells of exogenous hormones. All experiments were performed in 24-well cell culture plates (Corning, Corning Incorporated, New York) pre-coated with 0.1% sterile gelatin solution. A 1 mL of cell suspension in DMEM/F-12 without phenol red (containing 15% charcoal-stripped foetal bovine serum, 2% HEPES, and 1% penicillin-streptomycin) at a concentration of 2×10^5 cells/mL was added to each well and the cells were allowed to attach for 24 h at 37 °C in a humidified atmosphere (95%) with 5% CO_2. After cell attachment, the media were changed and the experiment was commenced. Cells were exposed to individual mycotoxins concentrations from 0.1 μM to 16 μM; DON, ZEN, α-ZOL, β-ZOL and OTA, and *p,p′*-DDT pesticides (0.1–16 μM) diluted in DMEM/F-12 without phenol red (containing only 5% charcoal-stripped foetal bovine serum, 2% HEPES, and 1% penicillin-streptomycin) for 48 h in the same 24-well cell culture plates in duplicate. Binary mixtures (*p,p′*-DDT/ZEN, *p,p′*-DDT/α-ZOL, *p,p′*-DDT/β-ZOL, OTA/ZEN, OTA/α-ZOL**,** ZEN/α-ZOL, ZEN/β-ZOL and α-ZOL/β-ZOL) with concentrations varying from 0.1 μM to 8 μM were also prepared and both the individual and binary mixtures were tested on the cell line for 48 h. DMSO at 0.1%

and 0.2 % were used as vehicle controls while 3′,5′-cyclic adenosine monophosphate analogue, 8-Bromoadenosine 3′,5′-cyclic adenosine monophosphate (8-Br-cAMP: 50 μM) was used as positive control. The positive control 8-Br-cAMP activates adenylyl cyclase enzyme and stimulate cAMP production which indirectly upregulates steroidogenesis in Leydig cells (Dankers et al., 2013). After 48 h, the cell media supernatants were collected from each well and centrifuged to remove cell debris that may be present in the media. These were then transferred into plastic 2 mL vials and stored at -20 °C until ready for testosterone and progesterone analysis. Two independent exposures were carried out for each treatment.

2.5.2 Treatment of BeWo placental cells for hormone assay

Before the commencement of the experiment, BeWo cells were grown in T75 flasks with DMEM/F-12 media without phenol red and containing 10% charcoal-stripped foetal bovine serum, and 1% penicillin-streptomycin for at least 24 hours to starve the cells of exogenous hormones. Experiments were performed either in 24-well (for P4 and E2 assay) or 12-well (for β-hCG assay) cell culture plates (Corning, Corning Incorporated, New York). A 1 mL of cell suspension in DMEM/F-12 without phenol red (containing 10% charcoal-stripped foetal bovine serum, and 1% penicillin-streptomycin) at a concentration of 2×10^5 cells/mL (for P4 and E2 assay) or 5×10^4 cells/mL (for β-hCG assay) was added to each well and the cells were allowed to attach for 24 h at 37 °C in a humidified atmosphere (95%) with 5% CO_2. After cell attachment, the media were changed and the experiment was commenced. Cells were exposed to individual mycotoxins concentrations from 0.1 μM to 16 μM; DON, ZEN, α-ZOL, β-ZOL and OTA, and *p,p′*-DDT pesticides (0.1–16 μM) diluted in DMEM/F-12 without phenol red (containing only 5% charcoal-stripped foetal bovine serum, and 1% penicillin-streptomycin) for 48 h in the same 24-well cell culture plates in duplicate. Binary mixtures (*p,p′*-DDT/ZEN, *p,p′*-DDT/α-ZOL, *p,p′*-DDT/β-ZOL, DON/ZEN, DON/α-ZOL, DON/β-ZOL, ZEN/α-ZOL and α-ZOL/β-ZOL) with equimolar concentrations varying from 0.1 μM to 8 μM were also prepared and both the individual and binary mixtures were tested on the cell line for 48 h. DMSO at 0.1% and 0.2 % were used as vehicle controls while 8-Bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP: 1 mM) was used as positive control. The positive control 8-Br-cAMP activates adenylyl cyclase enzyme and stimulation of cAMP production which

indirectly upregulates steroidogenesis (Dankers et al., 2013). After 48 h, the cell media supernatants were collected from each well and centrifuged to remove cell debris that may be present in the media. These were then transferred into sterile plastic 2 mL vials and stored at -20 °C until ready for hormone analysis. Two independent exposures were carried out for each treatment with at least three internal repeats.

2.5.3 Progesterone

The DRG progesterone (P4) ELISA kit (EIA-1561) was used in the quantification of P4 in the cell culture media supernatant. The kit was developed for use in the detection of P4 in plasma or serum, but has been applied to accurately quantify P4 in cell culture media supernatant (Frizell et al., 2011). The assay range is 0 – 40 ng/mL and a sensitivity of approximately 60.045 ng/mL while the cross reactivity was 0.30% for 17α-hydroxylprogesterone and pregnenolone (0.35%). The manufacturer's instruction was followed except that the progesterone standards were prepared using DMEM/F12 media. Before the commencement of the experiment, the frozen cell media supernatant and the ELISA kit were allowed to thaw and reach room temperature. The ELISA wash buffer was prepared by diluting 6 mL of the concentrated wash solution (40x) with 234 mL of ultra-high purity water. The P4 standards (0 ng/mL, 0.3 ng/mL, 1.25 ng/mL, 2.5 ng/mL, 5.0 ng/mL, 15 ng/mL and 40 ng/mL) were prepared by diluting Cayman P4 stock standard (Cayman Chemical, Ann Arbor, USA; Cat. No.: 482604) with ultra purity water to 60 ng/mL bulk standard and this was serially diluted to get standards used for standard curve preparation. Then 25 µL of the standards and cell media supernatants were added to the designated wells in the microtitre plate and incubated for 5 minutes in the dark at room temperature. Thereafter, 200 µL of the enzyme conjugate was added to each well, mixed thoroughly for 10 seconds and incubated for 1 h at room temperature in the dark. After 1 h incubation, each well was inverted and washed three times using 400 µL of the wash buffer, tapped 4 to 5 times on absorbent paper towel to completely remove the liquid. Then 200 µL of the 3,3',5,5'-Tetramethylbenzidine (TMB) substrate was added to each well and the plate was incubated for 15 minutes at room temperature for the colour to develop. Thereafter, 100 µL of tetraoxosulphate (VI) acid (H_2SO_4) stop solution was added to each well to stop the enzymatic reaction and the absorbance was read at 450 nm wavelength using Multiskan FC® microtitre plate reader (Thermo Scientific, Vantaa, Finland) within 10 minutes. The P4 levels were

quantified in triplicate and results were calculated automatically using 4 parameters Logistics curve fit.

2.5.4 Testosterone

The Cayman's testosterone ELISA kit (Cayman Chemical, Ann Arbor, USA; Cat. No.: 582701) was used in the detection of testosterone concentration in the cell culture media supernatants after treatment following the manufacturer's instruction. The ELISA kit was developed for the analysis of testosterone level in plasma, serum, cell culture supernatant and other sample matrices with an assay range of 3.9 – 500 pg/mL and a sensitivity of approximately 6 pg/mL while the cross reactivity was 27. 4% for 5α-dihydrotestosterone, 18.9% for 5β-dihydrotestosterone, and 3.7% for androstenedione. The principle of the assay is that testosterone and testosterone-acetylcholinesterase (AChE) conjugate (testosterone tracer) compete for a limited amount of testosterone antiserum. Before the commencement of the experiment, the frozen cell media supernatant and the ELISA kit were allowed to thaw and reach room temperature. The ELISA wash buffer was prepared by diluting 10 mL of ELISA buffer concentrate (10x; Cat. No.: 400060) with 90 mL of ultra-high purity water (UPH, 18 MV/cm). Also the ELISA wash buffer was prepared by by diluting 5 mL of wash buffer concentrate (400x; Cat. No.: 400062) with 1,995 mL of UPH and the addition of 1 mL polysorbate 20 (cat. No.: 400035). The testosterone AChE tracer (Cat. No.: 482700) and testosterone ELISA antiserum (Cat. No.: 482702) were reconstituted by adding 6 mL of the ELISA buffer to each of them. In addition, 60 µL of the tracer dye and 60 µL of anti-serum dye were added to the testosterone AChE tracer and testosterone ELISA antiserum, respectively and mixed gently to dissolve. The testosterone ELISA standards (500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.3 pg/mL, 15.6 pg/mL, 7.8 pg/mL and 3.9 pg/mL) were prepared by diluting 100 µL of the 50 ng/mL stock standard (Cat. No.: 482704) with 900 µL of ultra pure water to obtain 5 ng/mL bulk stock standard and serially diluting the 5 ng/mL bulk stock standard with phenol-free DMEM/F12 media used for mycotoxin and pesticide dilution during BeWo cell or MA-10 cell treatment. Then 50 µL phenol-free DMEM/F12 media was added to the two non-specific binding wells (NSB) and two maximum binding wells (B_0) followed by the addition of 50 µL of ELISA buffer to the NSB wells. Thereafter, 50 µL of the testosterone standards and samples were added to the designated wells. Then 50 µL of the testosterone AChE

tracer was added to all the wells, except the blank (BLK) and total activity (TA) wells. This was followed by the addition of 50 µL of testosterone ELISA antiserum to all the wells, except the TA, NSB, and the BLK wells. The plate was then covered with plastic ELISA plate sealer and incubated for 2 h at room temperature on an orbital plate shaker in the dark. After 2 h incubation, each well was inverted and washed five times using 400 µL of the wash buffer, tapped 4 to 5 times on absorbent paper towel to completely remove the liquid. Then the Ellman reagent (Cat. No.: 400050) was reconstituted with 20 mL ultra pure water immediately and 200 µL was added to each well followed by the addition of 5 µL of AChE tracer to the TA well. The plate was covered with ELISA plate sealer and incubated for 60 – 90 minutes on an orbital shaker in the dark of colour development. Thereafter, the plate was read at 405 nm wavelength using Multiskan FC® microtitre plate reader (Thermo Scientific, Vantaa, Finland) within 10 minutes. The intensity of the colour is proportional to the amount of testosterone tracer bound to the well and this is inversely proportional to the amount of free testosterone in the well during incubation. The T levels were quantified in triplicate and results were calculated using 4 parameters Logistics curve fit or Cayman ELISA data analysis spread sheet.

2.5.5 Oestradiol

The Cayman's oestradiol (E2) ELISA kit (Cayman Chemical, Ann Arbor, USA; Cat. No.: 582251) was used in the detection of E2 concentration in the cell culture media supernatants after treatment following the manufacturer's instruction. The ELISA kit was developed for the analysis of E2 level in plasma, serum, cell culture supernatant and other sample matrices with an assay range of 6.6 – 4,000 pg/mL and a sensitivity of approximately 15 pg/mL while the cross reactivity to oestradiol-3-sulfate was 14.5%, oestradiol-3-glucuronide (14%), estrone (12%) and oestradiol-17-glucuronide (10%). The principle of the assay is that oestradiol and oestradiol-acetylcholinesterase (AChE) conjugate (oestradiol tracer) compete for a limited amount of oestradiol antiserum. Before the commencement of the experiment, the frozen cell media supernatant and the ELISA kit were allowed to thaw and reach room temperature. The ELISA wash buffer was prepared by diluting 10 mL of ELISA buffer concentrate (10x; Cat. No.: 400060) with 90 mL of ultra-high purity water (UPH, 18 MV/cm). Also the ELISA wash buffer was prepared by by diluting 5 mL of wash buffer concentrate (400x; Cat. No.: 400062) with 1,995 mL of UPH and the addition of 1 mL polysorbate 20 (cat. No.: 400035). The

oestradiol AChE tracer (Cat. No.: 482250) and oestradiol ELISA antiserum (Cat. No.: 482252) were reconstituted by adding 6 mL of the ELISA buffer to each of them. In addition, 60 µL of the tracer dye and 60 µL of anti-serum dye were added to the oestradiol AChE tracer and oestradiol ELISA antiserum, respectively and mixed gently to dissolve. The oestradiol ELISA standards (4,000 pg/mL, 1,600 pg/mL, 640 pg/mL, 256 pg/mL, 102.4 pg/mL, 41.0 pg/mL, 16.4 pg/mL and 6.6 pg/mL) were prepared by diluting 100 µL of the 400 ng/mL stock standard (Cat. No.: 482254) with 900 µL of ultra pure water to obtain 40 ng/mL bulk stock standard and serially diluting the 40 ng/mL bulk stock standard with phenol-free DMEM/F12 media used for mycotoxin and pesticide dilution during BeWo cell treatment. Then 50 µL phenol-free DMEM/F12 media was added to the two non-specific binding wells (NSB) and two maximum binding wells (B_0) followed by the addition of 50 µL of ELISA buffer to the NSB wells. Thereafter, 50 µL of the standards and samples were added to the designated wells. Then 50 µL of the oestradiol AChE tracer was added to all the wells, except the blank (BLK) and total activity (TA) wells. This was followed by the addition of 50 µL of E2 ELISA antiserum to all the wells, except the TA, NSB, and the BLK wells. The plate was then covered with plastic ELISA plate sealer and incubated for 1 h at room temperature on an orbital plate shaker in the dark. After 1 h incubation, each well was inverted and washed five times using 400 µL of the wash buffer, tapped 4 to 5 times on absorbent paper towel to completely remove the liquid. Then the Ellman reagent (Cat. No.: 400050) was reconstituted with 20 mL ultra pure water immediately and 200 µL was added to each well followed by the addition of 5 µL of AChE tracer to the TA well. The plate covered with ELISA plate sealer and incubated for 60 – 90 minutes on an orbital shaker in the dark of colour development. Thereafter, the plate was read at 405 nm wavelength using Multiskan FC® microtitre plate reader (Thermo Scientific, Vantaa, Finland) within 10 minutes. The intensity of the colour is proportional to the amount of oestradiol tracer bound to the well and this is inversely proportional to the amount of free E2 in the well during incubation. The oestradiol levels were quantified in triplicate and results were calculated using 4-parameters Logistics curve fit or Cayman ELISA data analysis spread sheet.

2.5.6 Beta-human chorionic gonadotropin

The DRG beta-human chorionic gonadotropin (β-hCG) ELISA kit (EIA-1911) is an enzyme immunoassay for the quantitative measurement of total human chorionic gonadotropin (hCG and β-hCG) and this was was used in the quantification of hCG and β-hCG in the cell culture media supernatant. Before the commencement of the experiment, the frozen cell media supernatant and the ELISA kit were allowed to thaw and reach room temperature. The lyophilised β-hCG standards (0, 5, 25, 50, 100, 200 mIU/mL) were reconstituted in 1 mL of ultra pure water. Then 25 µL of the standards and cell media supernatants were added to the designated wells in the microtitre plate and incubated for 5 minutes in the dark at room temperature. Thereafter, 200 µL of the enzyme conjugate is added to each well, mixed thoroughly for 10 seconds and incubated for 1 h at room temperature in the dark. After 1 h incubation, each well was inverted and washed five times using 400 µL of ultra pure water, tapped 4 to 5 times on absorbent paper towel to completely remove the liquid. Then 100 µL of the TMB substrate was added to each well and the plate was incubated for 15 minutes at room temperature in the dark for the colour to develop. Thereafter, 50 µL of H_2SO_4 stop solution was added to each well to stop the enzymatic reaction and the absorbance was read at 450 nm wavelength using Multiskan FC® microtitre plate reader (Thermo Scientific, Vantaa, Finland) within 10 minutes. The β-hCG levels were quantified in triplicate and results were calculated automatically using 4 parameters Logistics curve fit.

2.6 Gene expression assay in BeWo placental cells

2.6.1 Cell treatment

BeWo cells were counted as shown in **section 2.3.4** and seeded at 4 x 10^5 cells per 2 mL of phenol-free DMEM/F12 media (containing 10% charcoal-stripped foetal bovine serum) in each well of the 6 well plate (Corning, Corning Incorporated, USA) and allowed to attach overnight at 37 °C in a humidified atmosphere (95%) with 5% CO_2. Thereafter, the old media were removed and the cells were treated with 2 mL of ZEN (0.1 µM and 8 µM), *p,p'*-DDT (0.1 µM and 8 µM), equimolar concentrations of ZEN/ *p,p'*-DDT (0.1 µM and 8 µM), and 0.1% DMSO diluted in phenol-free DMEM/F12 media (containing 5% charcoal-stripped foetal bovine serum) in duplicate wells. After

48 h treatment, the media were removed, cells trypsinsed and duplicate wells of each treatment were pooled in the tubes. This was followed by centrifuging the tubes at 1200 RPM for 5 minutes and discarding the media supernatant. The cell pellets were resuspended in sterile PBS and cells were washed one more time to remove the media completely. Afterwards, the cells were ready for total RNA extraction.

2.6.2 RNA extraction

The Qiagen RNeasy Mini Kit (Qiagen, Crawley, UK) was used for the total RNA extraction from BeWo cells. After treatment, the harvested cell number was $\leq 6 \times 10^6$ for pooled duplicate wells. Cells were mixed with 350 µl of RLT buffer for 15 minutes at room temperature with gentle shaking to allow for complete lysis of the cells. Then, 350 µL of 70% of ethanol was added to the lysate and mixed well by pipetting. Approximately 700 µL of each sample was transferred to an RNeasy Mini spin column placed in a 2 mL collection tube. The tubes were centrifuged at 4 °C for 1 min at 4,000 x g, and 5 seconds at 6,000 x g. The flow through was discarded. This was followed by the addition of 700 µL buffer RW1 to each lysate in the RNeasy Mini spin column. The tubes were centrifuged at 4 °C for 1 min at 4,000 x g, and 5 seconds at 6,000 x g. The flow through was discarded. Then, 500 µL of buffer RPE was added to each RNeasy Mini spin column. The tubes were centrifuged at 4 °C for 1 min at 4,000 x g, and 5 seconds at 6,000 x g. The flow through was discarded. Another 500 µL of buffer RPE was added to each spin column. The tubes were centrifuged at 4 °C for 2 min at 4,000 x g, and 5 seconds at 6,000 x g. The flow through was discarded. Thereafter, the tubes were centrifuged again at 4 °C for 90 seconds at the maximum centrifugal speed to dry the membranes. The 2 mL collection tubes were discarded and replaced by the new 1.5 mL collection tubes. Finally, 30 µL of RNase/DNase free water was added to each spin column membrane. The tubes were centrifuged at 4 °C for 1.5 min at 6000 x g to elute the total RNA. The quantity and quality of total RNA from each sample was measured by NanoDrop 1000 Spectrophotometer V3.0.1 (Thermo Scientific, UK). The 260/280 values ranged from 1.8 – 2.0 while the 260/230 values ranged 2.0 – 2.2. The total RNA samples were converted to cDNA or stored at - 80 °C.

2.6.3 Complementary deoxyribonucleic acid (cDNA) synthesis

The reverse transcription of total RNA to cDNA was performed using Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, UK; Cat. No: 4374966). The total RNA samples were diluted to 50 ng/µL with RNase/DNase free water. Each PCR reaction was made up to 20 µL of the reaction volume. The enzymes were kept in the freezer until immediately prior to use and placed on ice during use. The 2X reverse transcription (RT) master mix were prepared as shown (**Table 2.3**), placed on ice and mixed gently. Then 10 µL of the 2X RT master mix was dispensed into each tube followed by the addition of 10 µL of RNA sample into each well, pipetting up and down twice, and then each tube was capped tightly. The mixture was centrifuged at at 4 °C to spin down the contents and eliminate any air bubbles that may have been trapped in the reaction mixture. Then the tubes were moved to the Veriti 96 Well Thermal Cycler (Applied Biosystems, UK) PCR machine for incubation under the following programmed conditions: Step 1 (25°C, 10 minutes), Step 2 (37 °C, 120 minutes), step 3 (85°C, 5 minutes) and Step 4 (indefinitely).

Table 2.3: Preparation of 2X RT master mix

Component	Volume (µL)
10X RT Buffer	2.0
25X dNTP Mix (100 mM)	0.8
10X RT Random Primers	2.0
Multiscribe^{TM} Reverse Transcriptase	1.0
RNase Inhibitor	1.0
Nuclease (RNA/DNA)-free water	3.2
Total per reaction	10.0

2.6.4 Quantitative real-time polymerase chain reaction (RT-qPCR) assay

The real time polymerase chain reaction is usually employed in molecular biology works to amplify and quantify DNA or RNA in a specimen preparation. Specific pre-determined oligonucleotides can amplify sequences in a DNA or cDNA template from

thousands to millions fold. The major principle is that PCR amplifies DNA exponentially, doubling the number of target molecules in each amplification cycle. Fluorescent DNA-binding dyes (e.g. SYBR green) and real time PCR machine are used to quantify the amplified products by measuring the fluorescence of the dyes and this yield increasing fluorescent signal in direct proportion to the number of PCR products generated. RT-qPCR method was used to quantify the differential gene expression by beWo cells after chemical treatment.

The primer for each gene used in the study was designed and synthesised by 2BScientific and Real Time Primers, LCC (Elkins Park, PA 19027) using their proprietary design methods. All primer sequences are shown in Appendix 2. The specificities of the primer pairs were checked using the nucleotide primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast) as described by Ye et al. (2012). The Applied Biosystems High Capacity cDNA transcription kit, gene primers and All-in-One^{TM} qPCR mix used in this study have been previously used in several human cell lines including BeWo trophoblast cell line (McGrane, 2015) and two different human embryonal carcinoma cell-lines (Christoforou, 2015) in order to assess the effect of different cell culture media on the expression of the selected genes in these cell lines. Christoforou, (2015) and McGrane, (2015) optimised and validated the primer efficiency and specificity for the majority of the primers used in the current thesis. Briefly, to check the primer efficiency and specificity, dilutions of the cDNA from reference samples (neat, 1:2, 1:5 and 1:10) and one negative control (DNase/RNase-free water and PCR master mix), were run in a PCR machine after which the PCR products were run at least once on an agarose gel. Importantly, single bands of the correct size were observed for each gene assay, that provides a clear indication that Ct values were derived from this one band, that contamination was unlikely and that genomic DNA was not amplified by each of the respective assays and that primer-dimers were absent. It was demonstrated that each RT-qPCR reaction produced a single band of the expected size and no additional bands or excessive levels of primer-dimer products were detected in any of the amplified cDNA samples. Further, since 96 different gene assays are used on the PCR array, if DNA contamination was present in the sample RNA/cDNA, this would have been clearly evidenced by a high and positive Ct value across every assay in the array, yet this was never observed.

Gene-specific primers (forward and reverse primers) were purchased from 2BScientific (Heyford Park, UK) in dry form in 96 well plate and at a final concentration of 50 µM in 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA when reconstituted in 20 µL RNase/DNase free water. Reconstituted plates were stored at -20 °C, and prior to use, the primers were allowed to thaw and diluted with RNase/DNase free water to 10 µM stock PCR plate. This was further diluted to 0.4 µM working plate and 5 µL was dispensed to each well of the PCR 96 well plate. A RT-qPCR master mix was prepared using 795 µL of All-in-One^{TM} qPCR mix (GeneCopoeia, USA), 32 µL of Rox dye (GeneCopoeia, USA), 227 µL of RNase/DNase free water and 20 µL of the cDNA. Then 10 µL of the RT-qPCR master mix was added to each well of the 96 well plate containing 5 µL of 0.4 µM gene-specific primers. The plate was placed on dry ice and centrifuged at 700 g at 60 seconds to remove any trapped air bubbles before being placed in the ABT PRISM 7900HT Sequence detection system (Applied Biosystems, UK) and programmed using the Sequence Detection System (SDS) software version 2.2. In the preliminary study, the expression of all the genes (**Appendix 2**) were run after treatment with toxins and some of the genes without significant fold difference were not taken further. Each reaction was ran in triplicate and a negative control (RNAase/DNase-free water) routinely included. Six references genes (*RPLP0, HPRT1, PGK1, GAPDH, YWHAZ* and *ACTB*) were tested for stability and *GAPDH* and *RPLP0* gave the best stable combinations of genes, and these were selected as reference genes for this study. The RT-qPCR reaction ran for 40 cycles and programmed thus: Stage 1 (50°C, 2 minutes), Stage 2 (95°C, 10 minutes), Stage 3 (95°C, 15 seconds), Stage 4 (58°C, 1 minute). The cycle threshold (C_T) values of each gene from the SDS. 2.2 programme were exported to excel spread sheet for processing and analysis. The ΔC_T was calculated from the difference expression between the genes of interest and the mean expression of the reference genes (GADPH and RPLPO). The $\Delta\Delta C_T$ was calculated from the difference in ΔC_T between cells exposed to the solvent control and mycotoxins/pesticides. The relative expression (fold change) was calculated using $2^{-\Delta\Delta C_T}$ as described by Livak and Schmitten (2001). The following equation was used in calculating the fold change in mRNA expression in the RT-qPCR: (i.e. $\Delta C_T = C_{T(\text{target gene})} - C_{T(\text{house keeping gene})}$; $\Delta\Delta C_T = \Delta C_{T(\text{sample})} - \Delta C_{T(\text{vehicle control})}$ and relative fold change (RFC) = $2^{-\Delta\Delta C_T}$. Relative fold change value was set up as 1 for vehicle control in the BeWo cells.

2.7 Statistical analysis

2.7.1 Analysis of MMV-Luc Cytotoxicity and Reporter Gene Assay data

Both RGA and MTT assay exposures were carried out in triplicate wells and in three independent experiments. Results were expressed as the mean ± standard deviation (Mean±SD) of the triplicate exposures. For the RGAs, relative dose response (fold induction) was calculated from the relative light units (RLUs) when compared with the negative control (*n*-fold) using Microsoft Excel (Microsoft, Redmond, Washington) and normalised with the response of E_2 (10 nM) arbitrarily set at 100%. The dose-response curves were fitted to a non-linear regression (four parameter curve fit) using GraphPad PRISM software version 6.0 (San Diego, CA) with a continuous log x axes. A one way analysis of variance (ANOVA) and Dunnett's multiple comparison test was used to determine significant differences between the treatments and the corresponding controls in the RGAs and MTT assays. A p value of < 0.05 was considered statistically significant, $p = \leq 0.05$ (*), ≤ 0.01 (**) and ≤ 0.001 (***).

The toxins interactions for the combined toxins on ER response were calculated as described in the formulae below.

Mean ER response of binary mixtures (expected in % of substance 1 + substance 2) = mean ER response (substance 1 in %) + mean ER response (substance 2 in %) - maximal ER response by 10 nM of E2 (100%).

The standard deviation (SD) was calculated using the model described by Weber et al. (2005) as shown below:

Expected SD (substance 1 + substance 2) = $[(\text{SD of substance 1})^2 + (\text{SD of substance 2})^2]^{1/2}$

In order to evaluate the interactive effects of the combined toxins below or above additivity, expected additive values were compared to actually measured values using a multiple t-test and corrected for multiple comparison using Holm-Šídák test method, with $p < 0.05$.

The results were interpreted as follows:

- **Additive effects:** measured ER response values were not significantly above or below the expected values.

- **Synergistic effects:** measured ER response values were significantly below expected values.
- **Antagonistic effects:** measured ER response values were significantly above expected values.

2.7.2 Analysis of the cytotoxicity assay data for MA-10 and BeWo cell lines

Each toxin treatment for the cytotoxicity assay for both MA-10 and BeWo cell lines was done in triplicates in three independent experiments. The reported values are mean viability (%) ± standard deviation (Mean±SD) with 0.1% DMSO (for single toxin treatment) or 0.2% DMSO (for binary combinations) as vehicle control. Data were analysed with GraphPad PRISM software version 6.0 (San Diego, CA). Differences between groups were analysed by one-way ANOVA followed by Dunnett's procedure for multiple comparisons. Significant effects are represented by $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****). The half maximal inhibitory concentration (IC_{50}) for the different compounds were determined by fitting dose-response curves to a non-linear regression (four parameter curve fit) using GraphPad PRISM software version 6.0 (San Diego, CA) with a continuous log x axes..

2.7.2.1 Calculation of expected cell viability

The expected cell viability of binary mixtures was calculated through using the arithmetic definition of aditivity which involves the addition of the mean viability (%) after exposure to one toxin with the mean viability (%) after exposure to second toxin and then substracted from the mean cell viability of the control. Toxins interactions were calculated as described by Weber et al. (2005) and Clarke et al. (2014) as shown below.

The model is as follows:

Mean viability of binary mixtures (expected in % of substance 1 + substance 2) = mean viability (substance 1 in %) + mean viability (substance 2 in %) - mean viability of control (100%).

2.7.2.2 Calculation of standard deviation of measured and expected values

The standard deviation (SD) was calculated using the model described by Weber et al. (2005) as shown below:

Expected SD (substance 1 + substance 2) = $[(\text{SD of substance 1})^2 + (\text{SD of substance 2})^2]^{1/2}$

In order to evaluate the effects and interactions of the combined toxins below or above additivity, expected additive values were compared to actually measured values using a multiple t-test and corrected for multiple comparison using Holm-Šídák test method, with $p < 0.05$.

The results were interpreted as follows:

- **Additive effects:** measured cell viability values were not significantly above or below the expected values.
- **Synergistic effects:** measured cell viability values were significantly below expected values.
- **Antagonistic effects:** measured cell viability values were significantly above expected values.

2.7.3 Analysis of hormone assay data

For the hormone assay, each toxin treatment was done in duplicates in two independent experiments. The reported values are mean testosterone (pg/mL) ± standard deviation (Mean ± SD), mean E2 (pg/mL) ± standard deviation (Mean ± SD), mean P4 (ng/mL) ± standard deviation (Mean ± SD) or mean of β-hCG (mIU/mL) ± standard deviation (Mean ± SD) with 0.1% DMSO (for single toxin treatment) or 0.2% DMSO (for binary combinations) as vehicle control. Difference in the hormone concentration of cells exposed to single toxins with the control were analysed using one-way ANOVA followed by Dunnett's procedure to correct for multiple comparisons. To determine the interactive effects of binary mixtures, the hormone concentrations were expressed as fold-induction compared to controls. The predictions for the combined treatments were made by assuming additive effects (Groten et al., 2001). In this model, it was assumed that FC_1 and FC_2 are the fold changes for exposure for single treatments 1 and 2, respectively. The additive model then predicts the fold change in their combined treatment to be $FC = FC_1 + FC_2 - 1$. Predicted expected values lower or greater than the measured values are regarded as antagonistic or synergistic, respectively. To test if the expected values were significantly different from the measured values, multiple t-test

was performed and correction for multiple comparison was done using Holm-Šídák test method. $p \leq 0.05$ was accepted as significant interactive effects.

2.7.4 Analysis of RT-qPCR assay data

For the gene expression assay, each toxin treatment was done in duplicates in three independent experiments. RT-qPCR was performed for the three independent experiments and assay for each gene of interest was done in three replicates in each plate. The fold change in mRNA expression was calculated as shown in section 2.6.4 above. The predictions for the combined treatments were made by assuming additive effects (Groten et al., 2001). As previously stated, it was assumed that FC_1 and FC_2 are the relative fold changes in mRNA expression for exposure for single treatments 1 and 2, respectively. The additive model then predicts the fold change in their combined treatment to be $FC = FC_1 + FC_2 - 1$. Data were analysed with multiple t-test and corrected for multiple comparison using Holm-Šídák test method. $p \leq 0.05$ was accepted as significant fold change and deviation from additive effects of binary mixtures.

Chapter 3

The effect of single and mixtures of mycotoxins and persistent organochloride pesticides on oestrogen receptor transcriptional activation using *in vitro* reporter gene assays

3.1 Introduction

Oestrogen (E2) plays an important role in cell growth, differentiation, and the proper functioning of both the male and female reproductive system (Guibourdenche et al., 2009; Prouillac and Lecoeur, 2010). These critical biological function of E2 are mediated through the oestrogen receptors (ER); ESR1 (ERα) and ESR2 (ERβ), which are members of the nuclear receptor superfamily. There has been increasing evidence that many natural chemicals in food and synthetic environmental chemicals have the ability to interfere with the ER activity in both humans and animals resulting in endocrine disruption (Connolly, 2009). Few natural toxins in food (e.g. mycotoxins) and some synthetic chemicals (e.g. pesticides) are known to possess estrogenic and/or anti-estrogenic properties and cause adverse reproductive health outcome in humans and animals, including precocious puberty and early thelarche in females, poor sperm quality, modified sexual behaviour, and alteration of the functions of reproductive organs in males and females (Diamanti-Kandarakis et al., 2009; Massart *et al*., 2008, 2010; Connolly et al., 2011; Bittner et al., 2014; Kowalska et al., 2016). ZEN and its metabolites also exhibited endocrine disrupting effects at the level of nuclear receptor signalling and steroidogenesis using *in vitro* bioassays (Frizzell et al., 2011).

Research into the effects of mycotoxins, including ZEN, α-ZOL and β-ZOL on ERs transcriptional activity has traditionally focused on the effects of single toxins (Kuiper et al., 1998; Shier et al., 2001; Frizzell et al., 2011; Cozzini et al., 2012; Molina-Molina et al., 2014; Ehrlich et al., 2015; Drzymala et al., 2015) while only a few studies have assessed the effects mixtures of these toxins may have on ERs transcriptional activity (Demaegdt et al., 2016; Vejdovszky et al., 2017a,b). It is well established that ZEN, α-ZOL and β-ZOL activate oestrogen receptor subtypes α and β both *in vitro* and *in vivo,* with α-ZOL showing the highest oestrogenic potency (EFSA, 2016; Tatay et al., 2018). The estrogenic potential of DDT and its metabolites have also been investigated with

p,p'-DDT and *p,p'*-DDE showing low agonistic activities on ERs whereas *o, p'*-DDT (l,l,l-trichloro-2(p-chlorophenyl)-2-(o-chlorophenyl) ethane) had strong oestrogenic activity (Chen et al., 1997; Brennan et al., 2016). However, there is no report on the effect of binary combinations of ZEN and α-ZOL on E_2-mediated ER transcriptional activation. In addition, there is no report on the effect of mixtures of ZEN and α-ZOL with *p,p'*-DDT or *p,p'*-DDE on E_2-mediated ER transcriptional activation. There is the possibility of ZEN, α-ZOL and β-ZOL appearing in mixtures in biological systems as they are produced simultaneously by *Fusarium species* in corn stems (Minervini and Dell'Aquila, 2008). In farm animals, ZEN can be biotransformed either in the intestine, liver or granulosa cells by 3α- and 3β-hydroxysteroid dehydrogenase (3α- and 3β-HSD) to α-ZOL and β-ZOL (Minervini and Dell'Aquila, 2008). In humans, ZEN and α-ZOL are the most prevalent and can occur in mixtures in biological samples (Bandera et al., 2011; Shephard et al., 2013). In addition, ZEN and its metabolites can be carried-over in animals used for human consumption which can result to human exposure. For instance, ZEN, α-ZOL and β-ZOL accounted for the majority of total ZEN residues in farm animals (Dänicke and Winkler et al., 2015). Furthermore, as pesticides especially *p,p'*-DDT and *p,p'*-DDE are common contaminants of the environment and agricultural produce, co-occurrence with mycotoxins is inevitable (Romero-González et al., 2011; Akoto et al., 2013). Therefore, this warrants research into the effects of binary mixtures of ZEN and α-ZOL and/or *p,p'*-DDT and *p,p'*-DDE on E_2-mediated ER transcriptional response.

Several *in vitro* methods are employed in the assessment of oestrogenic and anti-oestrogenic effects of food and environmental toxins, and these falls into four categories, namely cell proliferation assays, receptor binding assay, *in vitro* gene expression assays and reporter gene assays. The major examples of these *in vitro* methods include E-screen using MCF-7 cell line (Soto et al., 1995; Tatay et al., 2018), ERα redistribution assay using recombinant osteosarcoma (U2OS) cells (Ehrlich et al., 2015), MMV-Luc reporter gene assay (Willemsen et al., 2004), ERα chemical activated luciferase gene expression (ERα CALUX ®) assay (van der Burg et al., 2010; Ehrlich et al., 2015), and ER ligand binding assay and transactivation assay (Kuiper et al., 1998). Other commonly used *in vitro* methods are upregulation of progesterone receptor (PR) mRNA expression in the Ishikawa cell line (Schaefer et al., 2010), alkaline phosphatase

activity in the Ishikawa cell line (Le Guevel and Pakdel, 2001; Vejdovszky et al., 2017a,b), recombinant yeast cells (Bovee et al., 2004), HeLa-9903 bioassay (US Environmental Protection Agency, 2009), and BG1LucER and BG1LucERbc9 transactivation bioassay (Rogers and Denison, 2000; Morisseau et al., 2009; Brennan et al., 2016). On the other hand, the *in vivo* oestrogenic activity of potential environmental and food chemicals has been generally assessed using the uterotrophic assay which is based on the increase in uterine weight or uterotrophic response in immature female rats as a result of ER-mediated water imbibition and cellular proliferation in the uterine tissue (Kleinstreuer et al., 2016). However, recent reports indicate that results from *in vitro* oestrogenic bioassays correlate greatly with data obtained from the *in vivo* uterotrophic assay (Wang et al., 2012; 2014a, b)

The aim of the present study was to evaluate the effect of single and mixtures of mycotoxins and persistent organochloride pesticides on E_2-mediated ER nuclear transcriptional activity using MMV-Luc reporter gene assay (MMV-Luc RGA). In reality, human beings are not exposed to single environmental or food oestrogens, but to mixtures with other oestrogens. Therefore, this study was concerned in understanding whether ZEN, α-ZOL, *p,p'*-DDT and *p,p'*-DDE plus E_2, or binary combinations of ZEN, α-ZOL, *p,p'*-DDT and *p,p'*-DDE plus E_2, could elicit an additive, synergistic or antagonistic effect on ER transactivation response.

3.2 Results

3.2.1 Cell viability and cytotoxicity

The single mycotoxins, pesticides and 17β-oestradiol (E_2) did not reduce the cell viability of MMV-Luc cell line after 48 h in all the concentrations tested (**Fig. 3.1**). It was found that ZEN and E2 significantly increased cell viability ($p \leq 0.001$) whereas α-ZOL increased cell viability at 1 μM and 50 μM ($p \leq 0.05$). In the concurrent exposure of either single or binary mixtures of mycotoxins and pesticides with E_2 at 0.05 nM and 10 nM, no cytotoxic effect was observed (**Fig 3.2 - 3.5**) indicating that the transcriptional responses observed are not as a result of reduced cell viability.

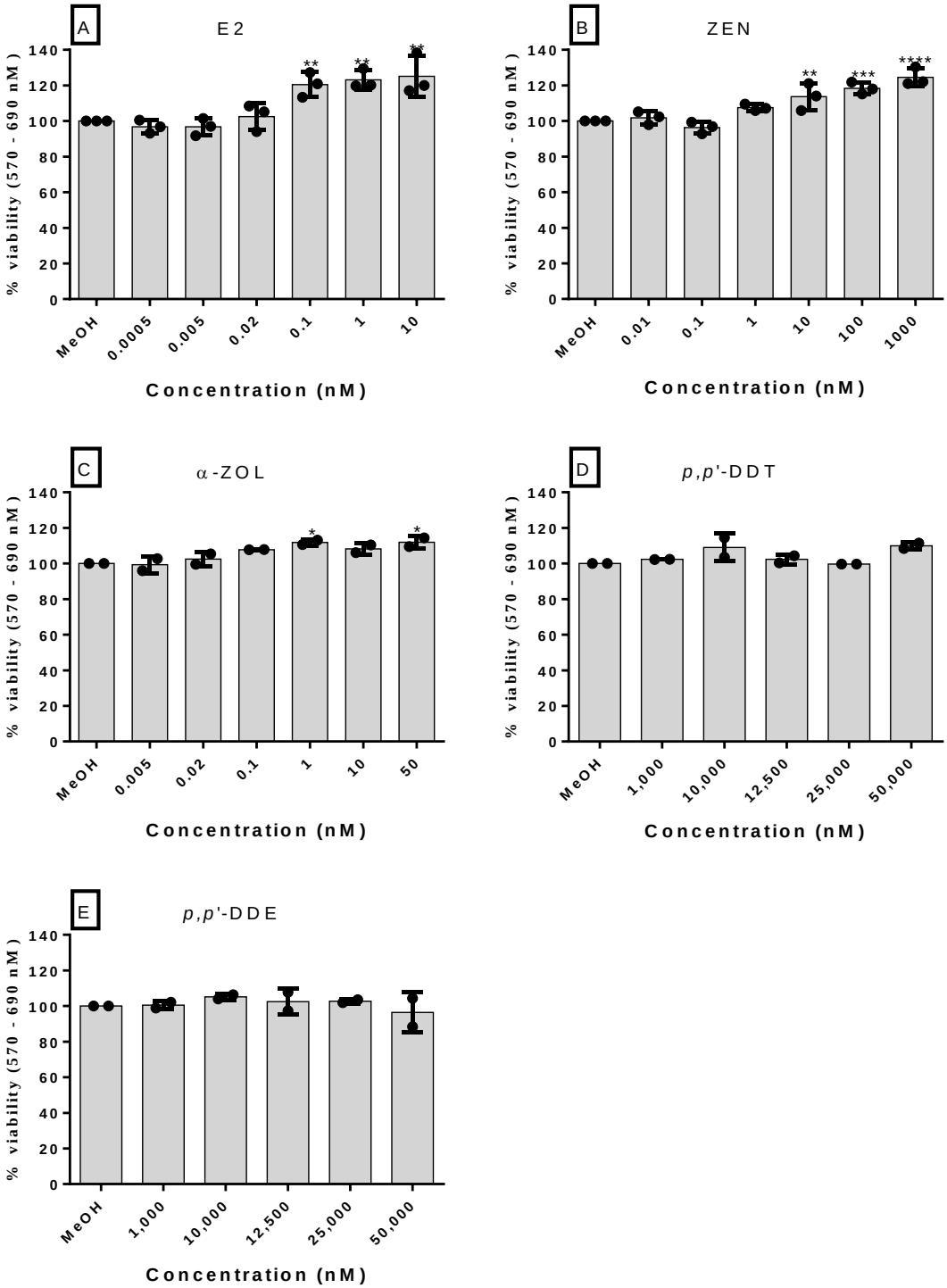

Fig. 3.1: Cell viability of MMV-Luc reporter gene cell line exposed to 17β-oestradiol (E_2), zearalenone (ZEN), alpha-zearalenol (α-ZOL), 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane (*p,p'*-DDT), and 1,1-dichloro-2,2-bis(p-chlorophenyl) ethylene (*p,p'*-DDE). Test substances were assayed in triplicate in at least two independent experiments. Data were analysed with one-way ANOVA and corrected for multiple comparison using the Dunnett's test method. Error bars represent standard deviation of

at least two biological replicates. Results are expressed as percentage of methanol (MeOH) control. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 and ****p ≤ 0.0001 represents significant effects.

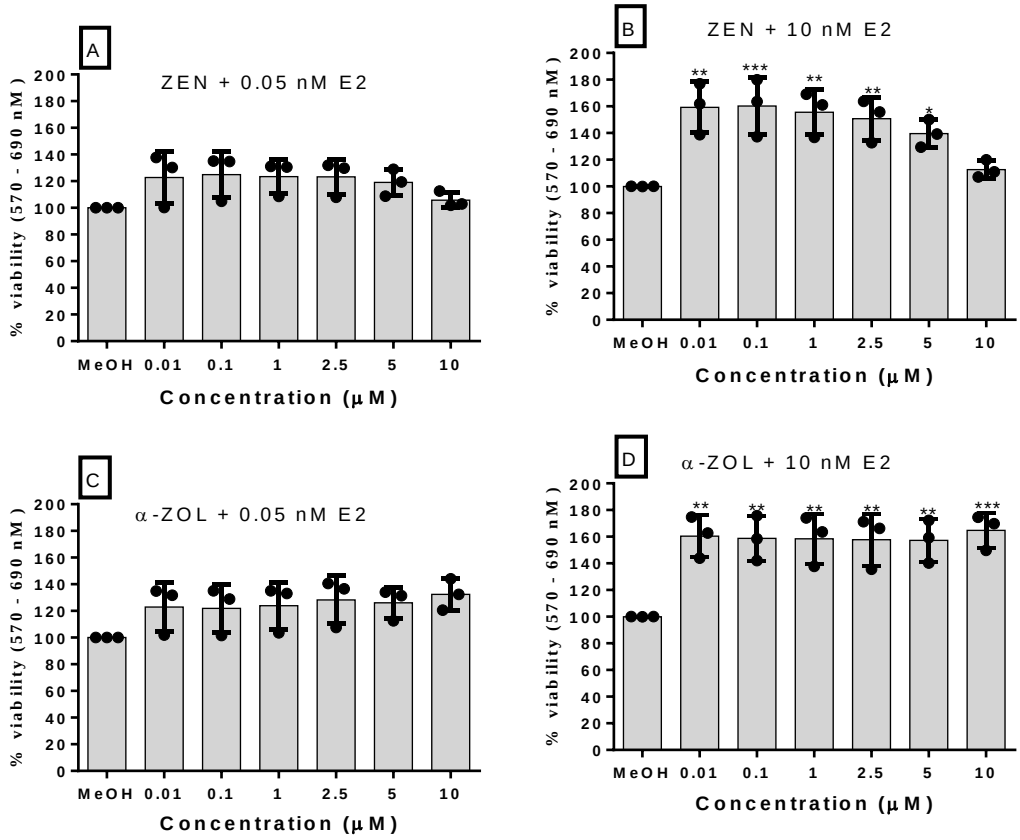

Fig. 3.2: Cell viability of MMV-Luc reporter gene cell line exposed to either zearalenone (ZEN) or alpha-zearalenol (α-ZOL) in combination with 0.05 nM or 10 nM of 17β-oestradiol (E_2). Test substances were assayed in triplicate in three independent experiments. Data were analysed with one-way ANOVA and corrected for multiple comparison using the Dunnett's test method. Error bars represent standard deviation of three biological replicates. Results are expressed as percentage of methanol (MeOH) control. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 and ****p ≤ 0.0001 represents significant effects.

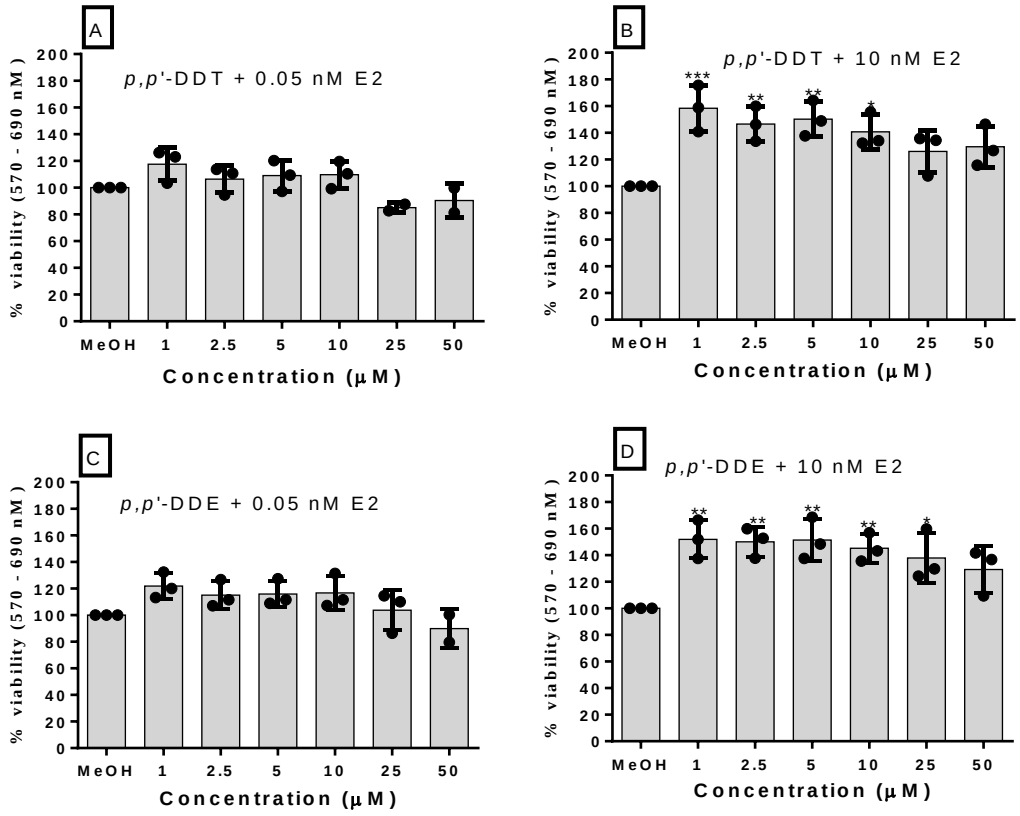

Fig. 3.3: Cell viability of MMV-Luc reporter gene cell line exposed to either 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane (*p,p'*-DDT), and 1,1-dichloro-2,2-bis(p-chlorophenyl) ethylene (*p,p'*-DDE) in combination with 0.05 nM or 10 nM of 17β-oestradiol (E_2). Test substances were assayed in triplicate in at least two independent experiments. Data were analysed with one-way ANOVA and corrected for multiple comparison using the Dunnett's test method. Error bars represent standard deviation of at least two biological replicates. Results are expressed as percentage of methanol (MeOH) control. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ and ****$p \leq 0.0001$ represents significant effects.

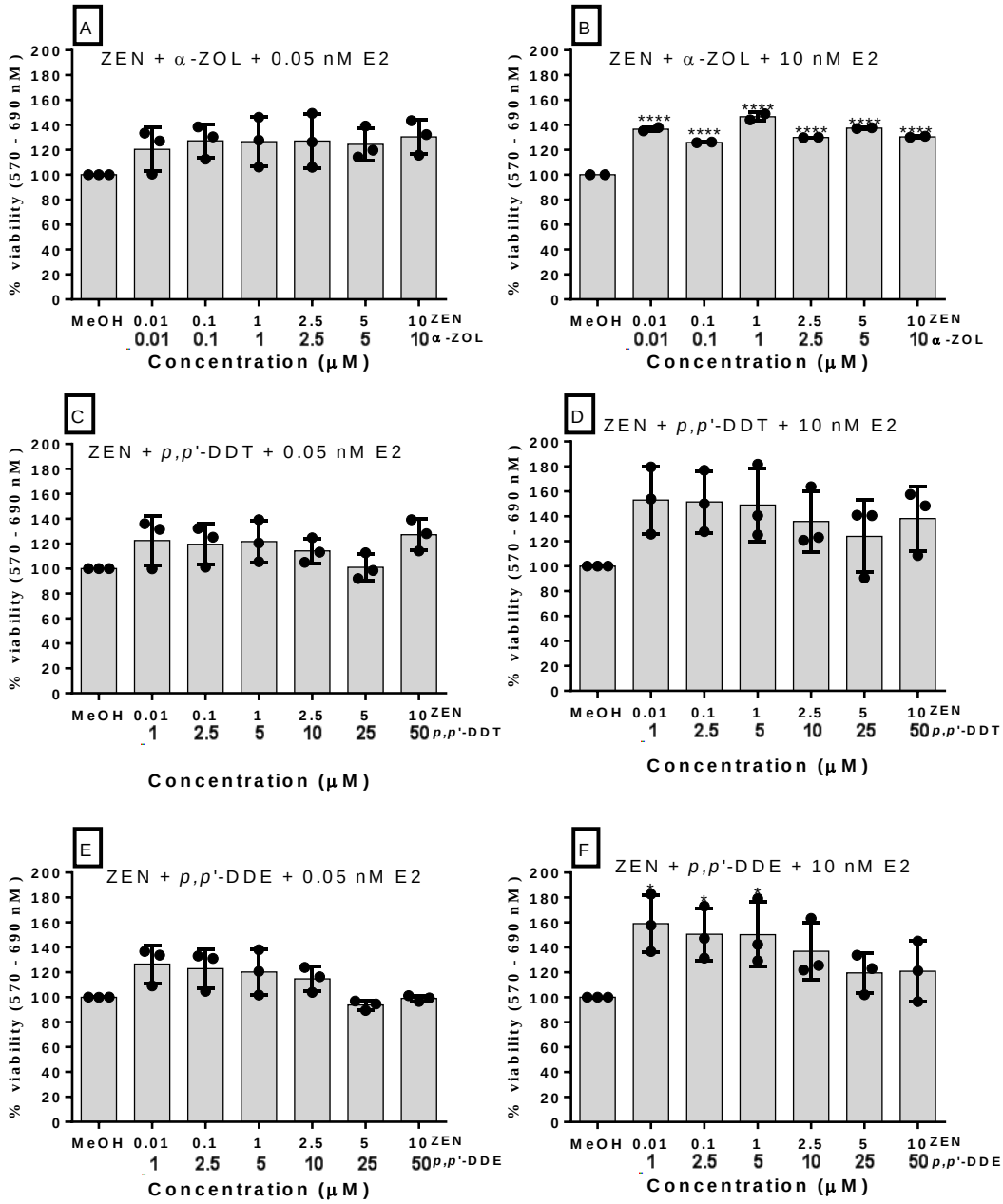

Fig. 3.4: Cell viability of MMV-Luc reporter gene cell line exposed to binary combination of zearalenone (ZEN) with either alpha-zearalenol (α-ZOL), 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane (*p,p'*-DDT), or 1,1-dichloro-2,2-bis(p-chlorophenyl) ethylene (*p,p'*-DDE) in conjunction with 0.05 nM or 10 nM of 17β-oestradiol (E_2). Test substances were assayed in triplicate in at least two independent experiments. Data were analysed with one-way ANOVA and corrected for multiple comparison using the Dunnett's test method. Error bars represent standard deviation of at least two biological replicates. Results are expressed as percentage of maximal induction of E_2 (0.05 nM or 10 nM). Results are expressed as percentage of methanol

(MeOH) control. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 and ****p ≤ 0.0001 represents significant effects.

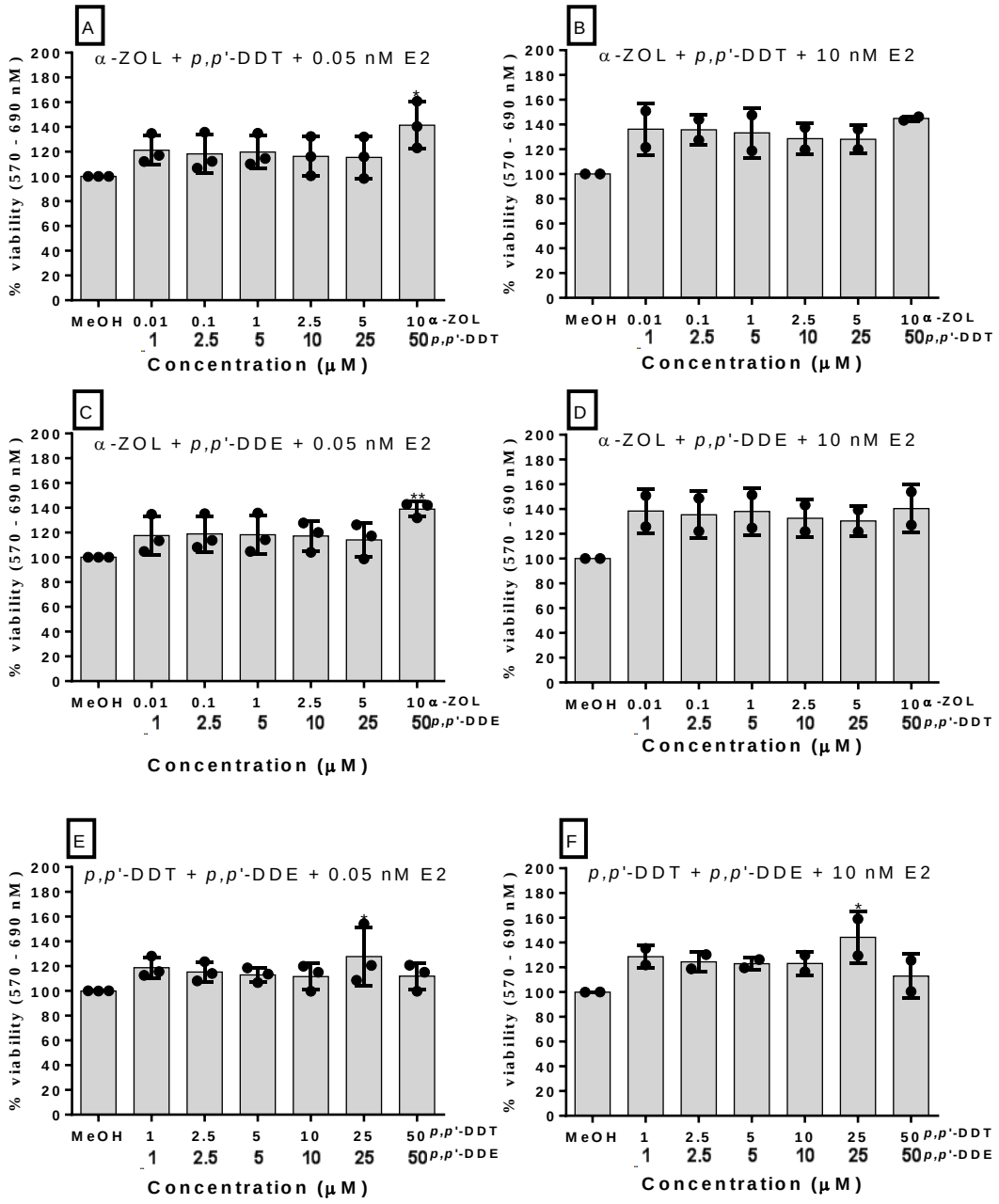

Fig. 3.5: Cell viability of MMV-Luc reporter gene cell line exposed to binary combination of α-ZOL with either *p,p'*-DDT (A & B), or *p,p'*-DDE (C & D), and co-exposure of *p,p'*-DDT and *p,p'*-DDE (E & F) in conjunction with 0.05 nM or 10 nM of 17β-oestradiol (E_2). Test substances were assayed in triplicate in at least two independent experiments. Data were analysed with one-way ANOVA and corrected for multiple comparison using the Dunnett's test method. Error bars represent standard deviation of at least two biological replicates. Results are expressed as percentage of

maximal induction of E_2 (0.05 nM or 10 nM). Results are expressed as percentage of methanol (MeOH) control. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 and ****p ≤ 0.0001 represents significant effects.

3.2.2 ER transcriptional response induced by single mycotoxins and persistent organochloride pesticides

The maximum induction of the E_2 (10 nM) standard in MMV-Luc reporter gene assay (RGA) was set at 100% in determining the transcriptional response of the other environmental and food-borne contaminants. Therefore, the response from other compounds is relative to this maximal response by 10 nM of E_2. The dose-response curve for the ER transcriptional activation of the single mycotoxins and pesticides is shown in **Figure 3.6**. Among the single compounds tested, E_2 at 1.0 nM activated ER transcriptional response closely to the maximum induction (95.93 ± 3.92%) (**Table 3.1**). The highest transcriptional activation for ZEN was at 1 µM (80.11 ± 5.58%) whereas its major metabolite α-ZOL at 50 nM induced similar response (73.45 ± 3.25%) (**Table 3.1**). A dose response curve was plotted (**Table 3.1**), E_2 had an EC_{50} of 0.053 ± 0.012 nM while ZEN and α-ZOL had an EC_{50} of 1.32 ± 0.10 nM and 0.27 ± 0.12 nM, respectively. In this study, *p,p′*-DDT and *p,p′*-DDE also weakly activated ER transcription with the highest response occurring at 50 µM, 28.70 ± 2.97% and 18.65 ± 1.77 %, respectively (**Table 3.1**).

3.2.3 Effects of single mycotoxins and persistent organochloride pesticides on E_2-mediated ER transcriptional response

This study further characterised the effects of mycotoxins and pestcides that were oestrogenic in the MMV-Luc RGA (ZEN, α-ZOL, *p,p′*-DDT and *p,p′*-DDE) and co-exposed each of them with E_2 (0.05 nM and 10 nM) to identify the effect of each of these toxins on the ER transcriptional response induced by E_2. It was observed that co-incubation of either ZEN or α-ZOL with E_2 (0.05 nM) significantly ($p \leq 0.001$) modulated the ER transcriptional response induced by E_2, alone (**Fig. 3.7A & 3.7B**) with more induction occurring at lower doses (0.01 µM - 2.5 µM) in each case. On the contrary, co-incubation of same doses of either ZEN or α-ZOL with 10 nM of E_2 caused a dose-dependent inhibition of ER response (**Fig. 3.7A & 3.7B**) reducing E_2-mediated

response by approximately 40% at 10 µM, in each case. This study also found that concurrent treatment of MMV-Luc cells with either *p,p′*-DDT or *p,p′*-DDE with E_2 (0.05 nM or 10 nM) significantly reduced the ER transcription activation caused by E_2, alone (**Fig. 3.7C & 3.7D**).

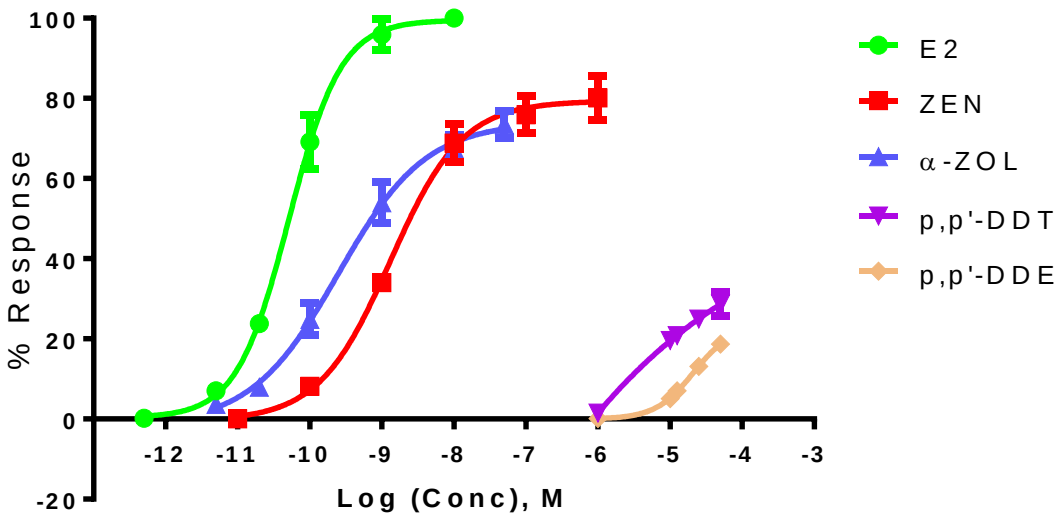

Fig. 3.6: Dose response curve of the transcriptional response elicited by 17β-oestradiol (E_2), zearalenone (ZEN), alpha-zearalenol (α-ZOL), 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane (*p,p′*-DDT), and 1,1-dichloro-2,2-bis(p-chlorophenyl) ethylene (*p,p′*-DDE) with MMV-Luc reporter gene cell line. Test substances were assayed in triplicate in three independent experiments. Error bars represent standard deviation from three biological replicates. Results are expressed as percentage of maximal induction of E_2 (100%; 10 nM).

Table 3.1: The EC_{50} and maximal ER transcriptional response of the tested mycotoxins and pesticides

	E2	**ZEN**	**α-ZOL**	***p,p'*-DDT**	***p,p'*-DDE**
EC_{50}	$5.30 \times 10^{-11} \pm 1.17 \times 10^{-11}$ M (0.053 ± 0.012 nM)	$1.32 \times 10^{-9} \pm 9.56 \times 10^{-11}$ M (1.32 ± 0.0956 nM)	$2.70 \times 10^{-10} \pm 1.21 \times 10^{-10}$ M (0.27 ± 0.121 nM)	N/A	N/A
R^2	0.9952	0.9905	0.9888	0.9845	0.9895
Maximal induction (%)	100 ± 0.00	80.11 ± 5.58	73.45 ± 3.25	28.70 ± 2.97	18.65 ± 1.77

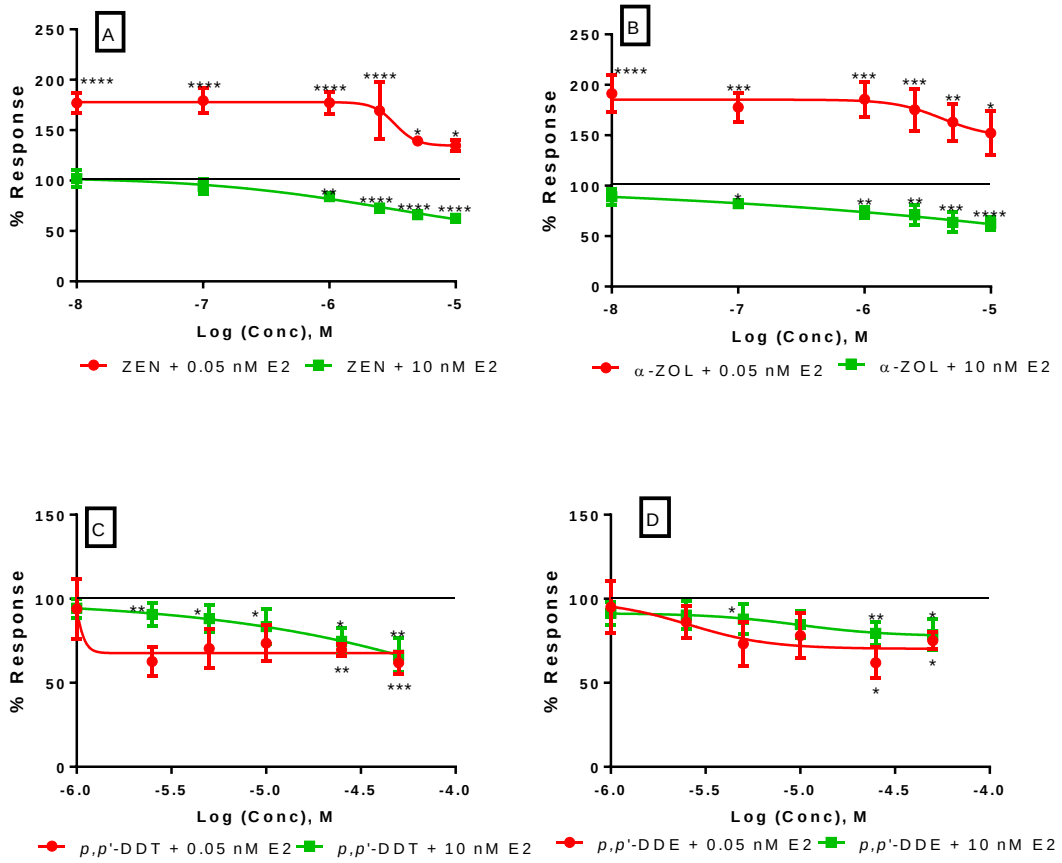

Fig. 3.7: ER transcriptional response induced when increasing concentrations of zearalenone (ZEN), alpha-zearalenol (α-ZOL), 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane (*p,p'*-DDT), or 1,1-dichloro-2,2-bis(p-chlorophenyl) ethylene (*p,p'*-DDE) were co-exposed with either 0.05 nM or 10 nM of E_2. Test substances were assayed in triplicate in three independent experiments. Results are expressed as percentage of maximal induction of E_2 (0.05 nM or 10 nM). Data were analysed with one-way ANOVA and corrected for multiple comparison using the Dunnett's test method. Error bars represent standard deviation of three biological replicates. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 and ****p ≤ 0.0001 represents significant effects.

3.2.4 Effects of binary mixtures of mycotoxins and persistent organochloride pesticides on E_2-mediated ER transcriptional response

Since human beings and wild life are exposed to endocrine disrupting compounds in mixtures rather than in single, this study also evaluated the effects of binary mixtures of the toxins (ZEN + α-ZOL, ZEN + *p,p'*-DDT, and ZEN + *p,p'*-DDE) with either 0.05 nM or 10 nM of E_2 to determine their effects on the transcriptional response induced by E2

at those concentrations (**Fig. 3.8**). Co-treatment of ZEN + α-ZOL, ZEN + *p,p′*-DDT, or ZEN + *p,p′*-DDE with E_2 (0.05 nM) significantly ($p \leq 0.001$) enhanced the transcriptional response induced by E2 alone at lower concentrations whereas little or no effects were observed at higher concentrations (**Fig. 3.8A, 3.8C & 3.8E**). In contrast, co-exposure of E_2 (10 nM) with ZEN + α-ZOL, ZEN + *p,p′*-DDT, or ZEN + *p,p′*-DDE significantly decreased the E2-mediated ER response dose-dependently ($p \leq 0.001$), with the highest doses combinations reducing ER transcriptional response to approximately 40% (**Fig. 3.8B, 3.8D & 3.8F**).

This study also tested the transactivation response that will be elicited by concurrent exposure of E2 (0.05 nM or 10 nM) with different dose combinations of α-ZOL + *p,p′*-DDT, α-ZOL + *p,p′*-DDE or *p,p′*-DDT + *p,p′*-DDE (**Fig. 3.9**). It was noted that α-ZOL + *p,p′*-DDT and α-ZOL + *p,p′*-DDE significantly ($p \leq 0.001$) modulated the transcriptional response of E2 at 0.05 nM, but strongly inhibited the ER response mediated by E2 (10 nM) with the highest dose combinations (α-ZOL: 10 µM; *p,p′*-DDT: 50 µM and *p,p′*-DDE: 50 µM) reducing E_2-mediated ER transactivation response by over 70% (**Fig. 3.9A – 3.9D**). Co-treatment of equimolar mixtures of *p,p′*-DDT and *p,p′*-DDE with E_2 at 0.05 nM or 10 nM caused a dose-dependent decrease in E_2-mediated ER transactivation response and this was completely inhibited at equimolar concentrations (50 µM) for both 0.05 nM and 10 nM of E_2, co-treatment (**Fig. 3.9E & 3.89**).

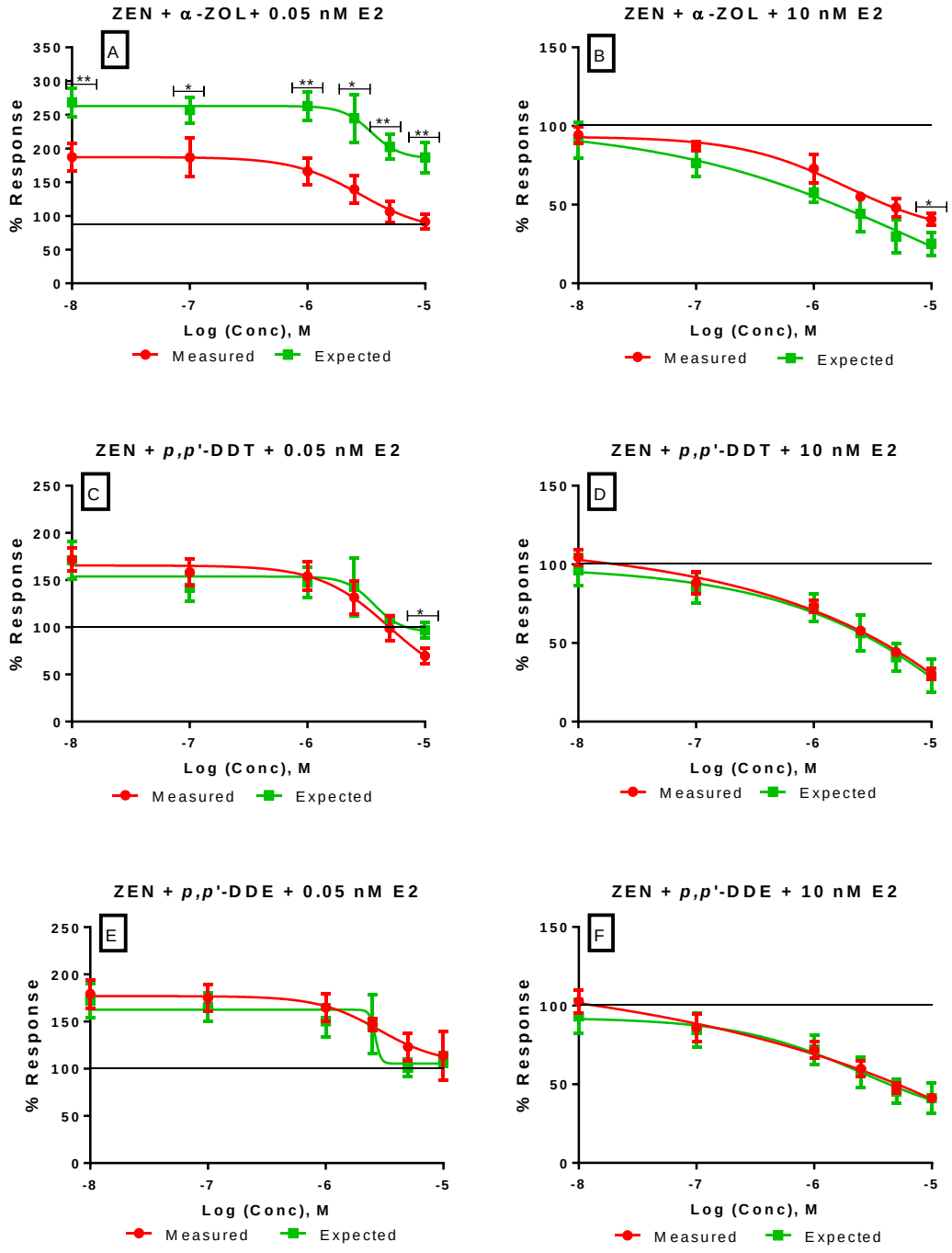

Fig. 3.8: Measured and expected ER transcriptional response induced when increasing concentrations of binary mixtures of mycotoxins and persistent organochloride pesticides were co-exposed with either 0.05 nM or 10 nM of E_2. Test substances were assayed in triplicate, in three independent experiments. Data were analysed with multiple t-test and corrected for multiple comparson using the Holm-Šídák test method. Error bars represent standard deviation of three biological replicates. Results are expressed as percentage of maximal induction of E_2 (0.05 nM or 10 nM). *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ and ****$p \leq 0.0001$ represents significant effects.

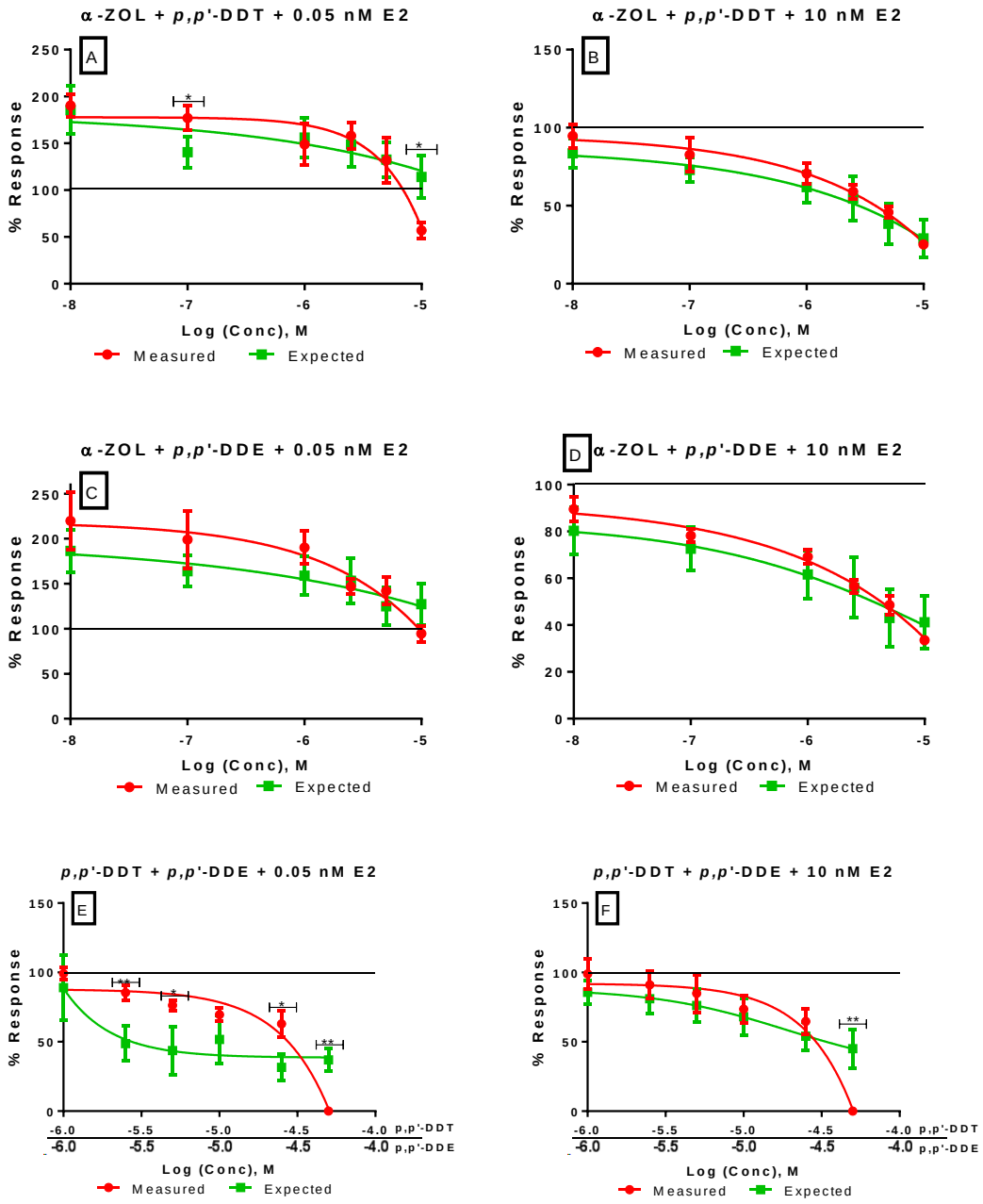

Fig. 3.9: Measured amd expected ER transcriptional response induced when increasing concentrations of binary mixtures of α-ZOL and persistent organochloride pesticides (*p,p′*-DDT and *p,p′*-DDE) were co-exposed with either 0.05 nM or 10 nM of E_2. Test substances were assayed in triplicate in three independent experiments. Data were analysed with multiple t-test and corrected for multiple comparson using the Holm-Šídák test method. Error bars represent standard deviation of three biological replicates. Results are expressed as percentage of maximal induction of E_2 (0.05 nM or 10 nM). *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 and ****p ≤ 0.0001 represents significant effects.

3.3 Discussion

There has been an increasing concern that environmental and dietary chemicals cause endocrine disruption leading to studies concerning their potential adverse effects on both animal and human reproduction (Diamanti-Kandarakis et al., 2009). In response to the European Union (EU) legislation on Registration, Evaluation, Authorisation and Restriction of chemical substances (REACH) and to fulfil the principle of Refinement, Reduction and Replacement of animal testing (3Rs), there have been an rising demand for the use of alternative *in vitro* and *in silico* methods in the characterisation of toxicity of chemicals in order to reduce the use of animal testing (Wang et al., 2014a, b; Ehrlich et al., 2015). As a result, several researches have shown that reporter gene assays are useful tools for the evaluation of the oestrogenic and anti-oestrogenic activity of single or mixtures of food and environmental contaminants, and could replace the *in vivo* uterotrophic assay (Connolly et al., 2011; Frizzell et al., 2011; Wang et al., 2014a, b; Ehrlich et al., 2015; Demaegdt et al., 2016). In this study, the MMV-Luc reporter gene assay was employed to determine the effects of single and mixtures of mycotoxins and persistent organochloride pesticides on oestrogen receptor transcriptional activity. The oestrogenic response obtained from the mammalian-based ER reporter gene cell lines has been shown to be well correlated with the response observed using the gold standard for oestrogenic assay *'in vivo* uterotrophic assay in immature or ovarietomised rodents' (Wang et al., 2014a, b), providing faster, easier and cheaper alternative testing strategy for oestrogenicity.

3.3.1 Cell viability and cytotoxicity

No cytotoxic effect was observed in the MMV-Luc cells within the range of mycotoxins and persistent organochlorides tested. Rather, the mycotoxins and persistent organochlorides either in single or mixtures with E_2 significantly increased cell viability depending on concentration and/or ratio of combination. Similarly, E_2 had no cytotoxic effect on the cells in any of the concentrations tested (0.001 - 10 nM), but significantly stimulated cell growth at 0.1 - 10 nM. This is in agreement to previous reports using this cell line (Frizzell et al., 2011; Ndossi et al., 2012; Frizzell et al., 2013; Demaegdt et al., 2016). Cells expressing ER, especially MCF-7 are known to be stimulated by oestrogenic chemicals and the MMV-Luc reporter cell line was derived from MCF-7

cell line (Shier et al., 2001; Frizzell et al., 2011; Molina-Molina et al., 2014; Ehrlich et al., 2015; Demaegdt et al., 2016; Tatay et al., 2018). The luminescence signal of a reporter gene assay can be decreased by an increase in cell cytotoxicity, therefore it is important to distinguish responses from interfering cytotoxic effects of chemicals. In addition, it must be noted that the increase in cell proliferation in treated cells contribute to higher luminescence response.

3.3.2 Transcriptional activation of ER by single mycotoxins and persistent organochloride pesticides

Among the single compounds tested, E_2 at 1.0 nM and 10.0 nM activated ER transcriptional response by approximately 95.93 ± 3.92% and 100% induction, respectively. ZEN induced approximately 80.11 ± 5.58% ER transcriptional response at 1 µM whereas its major metabolite α-ZOL induced approximately 73.45 ± 3.25% ER transcriptional response at 50 nM, relative to the response exhibited by 10 nM E_2 (100%). This is in agreement with the result of Ehrlich et al. (2015) who reported that ZEN and its metabolites (0.05 pM – 0.5 µM) induced maximum ER transcriptional response of 60 – 75% in human oestrogen receptor-chemically activated luciferase expression (hER-CALUX) assay. However, in human 293 embryonal kidney (HEK) cells expressing luciferase (Luc) and β-galactosidase (LacZ), the transcriptional response of ZEN on ERα and ERβ at 1 µM was 91% and 27%, respectively (Kuiper et al., 1998). The EC_{50} values of each compound were measured from a dose response curve and was used as a representative of oestrogenic potency. In this study, the natural oestrogen E_2 strongly induced oestrogenic response with an EC_{50} value of 0.053 ± 0.012 nM which is similar to most previously reported EC_{50} values of E_2 using different assay methods (Jobling et al., 1995; Balaguer et al., 1996; Sonneveld et al., 2005; Brennan et al., 2016). However, this EC_{50} value of E_2 (0.053 ± 0.012 nM) was slightly higher than the ones reported by Frizzell et al. (2011) and Demaegdt et al. (2016a) using the same oestrogenic responsive (MMV-Luc) cell line. It is possible incubation of chemicals in cells for longer duration (e.g. 48 h) compared to shorter duration (e.g. 24 h) could affect their oestrogenic potency possibly due to metabolic capacity of cells. The comparison of the EC_{50} data of ZEN and its major metabolite α-ZOL was also in agreement with previous studies with α-ZOL showing an increased oestrogenic potency compared with the parent compound ZEN (**Table 3.2**). Although the ranking of oestrogenicity of ZEN

and α-ZOL was similar to report of previous studies, the EC_{50} values (ZEN: 1.32 ± 0.0956 nM and α-ZOL: 0.27 ± 0.121 nM) varied from those reported by Frizzell et al. (2011) and Demaegdt et al. (2016) using the same reporter cell line. It has been shown that the use of antibiotics or antifungals in culture media contribute to high background levels of luciferase response in T47D-KBLuc reporter cell line (Wilson et al., 2004). This can also be corroborated by the work of Covaleda and co-workers which highlighted that the presence of tetracycline in assay media inhibited ERβ protein expression and modulated E_2-induced cell proliferation compared to that observed in the absence of tetracycline (Sotoca Covaleda et al., 2008). One major drawback of using the ER reporter assay over the activation of endogenous ER gene targets is that the stability of the luciferase reporter gene transfected into the cells could affect their luciferase responsiveness. In addition, unstable transfection can lead to a loss of the luciferase reporter gene construct from the genome of the cells resulting in variations in absolute luminescence values.

Table 3.2: Summary of the EC_{50} values (nM) of zearalenone (ZEN) and its metabolites reported from several *in vitro* bioassays

References	*In vitro* bioassay model	Bioreporter	Assay time	ZEN	α-ZOL	β-ZOL	α-ZAL	β-ZAL
Bovee et al. 2004	Yeast-based hER	yEGFP	4 - 24 h	130.0	11.0	230.0	18.0	230.0
Coldham et al., 1997	Yeast-based hER	LacZ	n/a	2.8	0.081	11.1	0.56	1.59
Cosnefroy et al., 2009	MELN hER	Luc	16 - 24 h	n/a	n/a	n/a	0.14	n/a
Cosnefroy et al., 2009	HELN-rtER	Luc	48 h	n/a	0.25	2.5	0.12	n/a
Demaegdt et al., 2016	MMV-luc (MCF-7 based)	Luc	24 h	0.87	0.031	13.0	n/a	n/a
Drzymala et al., 2015	MCF-7 proliferation	n/a	n/a	0.62	0.0011	11.0	0.046	1.2
Ehrlich et al., 2015	U2OS-hERα redistrubtion	EGFP	24 h	4.27	0.22	48.69	0.20	0.79
Ehrlich et al., 2015	hERα-CALUX	Luc	24 h	0.49	0.0096	2.5	0.09	0.28
Frizzell et al., 2011	MMV-luc (MCF-7 based)	Luc	24 h	1.6 ± 0.23	0.022 ± 0.001	3.9 ± 0.19	n/a	n/a

References	*In vitro* bioassay model	Bioreporter	Assay time	ZEN	α-ZOL	β-ZOL	α-ZAL	β-ZAL
Le Guevel and Pakdel, 2001	Yeast-based hER	LacZ	4 h	130.0 ± 20.0	30.0 ± 6.0	280.0 ± 40.0	40.0 ± 9.0	160 ± 10
Le Guevel and Pakdel, 2001	Ishikawa cell line alkaline phosphatase (ALP) activation	ALP	48 h	0.058 ± 0.01	0.0066 ± 0.001	25.0 ± 10	0.03 ± 0.006	0.32 ± 0.05
Le Guevel and Pakdel, 2001	Yeast-based rtER	LacZ	4 h	62.0 ± 8.0	12.0 ± 1.0	> 50,000	30.0 ± 7.8	110.0 ± 10.0
Malekinejad et al., 2005	MCF-7 proliferation	n/a	4 - 6 days	1.64	0.05	20.00	0.11	n/a
Minervini et al., 2005	MCF-7 proliferation	n/a	n/a	3.1	0.014	5.2	0.17	0.26
Molina-Molina et al., 2014	MCF-7 proliferation	n/a	6 days	3.81 ± 0.25	0.06 ± 0.01	8.49 ± 1.21	0.14 ± 0.04	2.29 ± 0.35
Pillon et al., 2005	MELN hER	Luc	16 h	0.313 ± 0.039	n/a	n/a	0.135 ± 0.031	n/a
Pinto et al., 2014	hERα-CALUX	Luc	16 h	n/a	0.13 ± 0.05	n/a	0.134 ± 0.074	n/a
Pinto et al., 2014	hERα-CALUX	Luc	16 h	n/a	0.44 ± 0.23	n/a	0.352 ± 0.13	n/a

References	*In vitro* bioassay model	Bioreporter	Assay time	ZEN	α-ZOL	β-ZOL	α-ZAL	β-ZAL
Shier et al., 2001	MCF-7 proliferation	n/a	5-7 days	1.1	0.012	2.5	0.06	0.31
Stypula-Trebas et al., 2015	Yeast-based hER	yEGFP	24 h	211.8 ± 38.7	20.0 ± 2.5	3603.0 ± 169.5	52.0 ± 0.2	449.2 ± 20.8
This study	MMV-luc (MCF-7 based)	Luc	48 h	1.341	0.265	n/a	n/a	n/a
Valimaa et al., 2010	Yeast-based hER	Luc	2.5 h	264.0	41.0	1890.0	62.0	515.0
Valimaa et al., 2010	Yeast-based hER	GFP	2.5 h	130.0	11.0	230.0	18.0	230.0
Vejdovszky et al., 2017a	Ishikawa cell line alkaline phosphatase (ALP) activation	ALP	48	1.423	0.055	n/a	n/a	n/a
Vejdovszky et al., 2017b	Ishikawa cell line alkaline phosphatase (ALP) activation	ALP	48 h	0.84 ± 0.06	n/a	n/a	n/a	n/a
Wang et al., 2014b	MCF-7/BOS proliferation	n/a	6 days	0.15	n/a	n/a	n/a	n/a

References	*In vitro* bioassay model	Bioreporter	Assay time	ZEN	α-ZOL	β-ZOL	α-ZAL	β-ZAL
Wang et al., 2014b	T47D ER-CALUX	Luc	24 h	0.23	n/a	n/a	n/a	n/a
Wang et al., 2014b	U2OS ERα-CALUX	Luc	24h	0.42	n/a	n/a	n/a	n/a
Wang et al., 2014b	Yeast-based hER	yEGFP	24 h	130.0	n/a	n/a	n/a	n/a

Key: HELN-rtER = human HeLa cells, rainbow trout oestrogen receptor; **MELN** = human MCF-7 cells, endogenous receptor; **Luc** = Luciferase; **LacZ** = Beta-galactosidase; **Cat** = Catalase; **yEGFP** = Yeast enhanced green fluorescent protein; **EGFP** = Enhanced green fluorescent protein; **n/a** = not available/not applicable; **Yeast-based rtER** = Saccheromyces cerevisae, rainbow trout oestrogen receptor; **CALUX** = Chemical Activated Luciferase Expression; **hER** = human oestrgen receptor

The organochloride pesticide *p,p′*-DDT and major metabolite *p,p′*-DDE showed weak oestrogenic response with the highest concentrations tested (50 µM) showing response of 28.70 ± 2.97% and 18.65 ± 1.77% relative to the maximal response induced by E_2 (100%) respectively. It was noted that *p,p′*-DDT significantly induced ER transcriptional response at 1 µM and above whereas significant response was only observed at 25 µM for *p,p′*-DDE. In a yeast-based reporter gene assay, *p,p′*-DDT and *p,p′*-DDE were ER agonists with *p,p′*-DDT significantly inducing the β-galactosidase activity at concentration of 1 µM and a 20 % response (EC_{20}) found at 51 µM (Li et al., 2008). In a more recent studies, *p,p′*-DDT (10 µM) induced an ER response of 19.0 ± 5% in an ERα expressing reporter (BG1LUc4E2) cell line and 30 ± 3 % in a reporter cell line ($BG1LucERB_C9$) expressing both ERα and ERβ (Brennan et al., 2016), similar to the results of this study. However, *p,p′*-DDE was a more potent agonist in a MELN reporter cell line with an EC_{50} of approximately 26 µM (Pillon et al, 2005) whereas *p,p′*-DDT only showed weak agonistic activity (7%) in HEK cells expressing ERα with luciferase reporter (Kuiper et al., 1998). In this study, DON and OTA had no oestrogenic response, similar to other studies using the same reporter cell line (Ndossi et al., 2012; Frizzell et al., 2013).

Oestrogens are essential in many reproductive function and can influence the growth, differentiation, and function of many target tissues in the reproductive system, including uterus, vagina, ovary, testes, epididymis, and prostate (Kuiper et al., 1998). In humans, about 1-2% of circulating E_2 is unbound while 40% is bound to sex hormone-binding globulin (SHBG) and the remaining fraction to albumin (JECFA; 2000). Currently, it is not clear whether ZEN and its metabolites interact or bind to carrier proteins (e.g. SHBG and albumin) as exhibited by endogenous hormones. In *in vitro* studies, the binding capacity of ZEN on SHBG from humans and cold-water fish Arctic charr (*Salvelinus alpinus*) was poor and therefore, the concentrations applied *in vitro* could have greater access to ER binding sites which may induce more response *in vivo* compared to endogenous oestrogens (Metzler et al., 2010; Molina-Molina et al., 2014). In addition, ZEN is known to activate the human pregnane X receptor (PXR), constitutive androstane receptor (CAR), and aryl hydrocarbon receptor (AhR) in HepG2 hepatoma cells, indicating that it can also mediate its effect through other nuclear receptors (Ayed-Boussema et al., 2001). Also worthy of note, it has been speculated that

ZEN and its metabolites, especially α-ZAL, α-ZOL and β-ZAL may act as androgen receptor (AR) antagonists as their half maximal inhibitory concentration (IC_{50}) values were within the same range as reported for two well-known androgen receptor antagonists vinclozolin and flutamide in various reporter gene assays (Molina-Molina et al., 2014; Stypula-Trebas et al., 2016). On the other hand, *p,p′*-DDE bioaccumulates in lipophilic tissues which are released into the blood and breast milk posing infant health risk as a result of its action as both ER and AR antagonists (Kelce et al., 1997). Therefore, it is possible that co-exposure of these mycotoxins and pesticides could lead to additive or synergistic effects on E_2-mediated ER activation.

3.3.3 Interactive effects of single mycotoxins and pesticides on E_2-mediated ER transcriptional activation

It was observed that co-treatment of ZEN or α-ZOL with 0.05 nM of E_2 significantly enhanced E_2-mediated ER response in MMV-Luc reporter cell line in all the doses tested. In a previous study using the same MMV-Luc reporter cell line, Wielogórska and co-workers (2015) showed that butyl 4-hydroxybenzoate (PB–Bu) and propyl 4-hydroxybenzoate (PB–Pr) which exhibited strong oestrogenic response also enhanced E_2-mediated response when co-exposed with 0.005 nM of E_2 (PB–Bu: 584.0% and PB–Pr: 460.0%). Also in agreement with the result of this study, several combinations of strong oestrogenic compounds ZEN and alternariol (AOH) or α-ZOL and AOH, at several concentrations had synergistic interaction and enhanced Ishikawa cell alkaline phosphatase production which exceeded the maximum induction (100%) reached by 1 nM of E2 (Vejdovszky et al., 2017a). This current study also observed that combination of ZEN (0.01 - 10 nM) and α-ZOL (0.01 - 10 nM) with the concentration of E_2 (10 nM), which showed maximal effect on ER transcriptional activation, resulted in significant dose-dependent reduction of E_2-mediated ER transcriptional response, indicating that combination of ZEN and α-ZOL act as partial agonists and competes with E_2 for ER. In a more recent study, Vejdovszky et al. (2017b) demonstrated that binary combinations of genistein with ZEN or AOH resulted in either synergism or antagonism in the alkaline phosphatase assay, depending on the combination ratios and the concentration range. They also posited that the nature of interactions between two strong oestrogenic compounds may depend on the concentration ratio of the substances in the mixture and also on the concentration range applied. Furthermore, Wielogórska et al. (2015)

demonstrated that the ER agonist di-*n*-butyl phthalate (DBP) at concentration of 1 µM had no significant effect the E_2-induced ER transcriptional activation when combined with E_2 at 0.1 nM, but exhibited antagonistic effect at 10 µM and 100 µM, reducing transactivation by 23% and 75%, respectively and this was not as a result of cytotoxic effect on cells. Therefore, mixtures of ZEN and α-ZOL with E_2 (0.05 nM) may be able to elicit a more potent oestrogenic or anti-oestrogenic response than initially anticipated, posing a greater risk to reproductive health.

This study also demonstrated that co-exposure of *p,p′*-DDT or *p,p′*-DDE (especially at ≥ 5 µM) with either 0.05 nM or 10 nM of E_2 significantly reduced the E_2-mediated ER transcriptional activity, indicating antagonistic effects (**Fig. 3.9E & 3.9F**). In a yeast-based hER reporter gene assay, combination of low dose of E_2 (0.3 nM) with low concentrations (40 nM - 1 µM) of *p,p′*-DDT, *o,p′*-DDT or o,p′-DDE caused dose-dependent increase in transcriptional response, but this was not significantly different from the sum of the separate responses observed in 0.3 nM of E_2 or DDT isomers, alone (Chen et al., 1997). In addition, co-treatment of the above DDT isomers at equimolar concentrations (40 nM and 80 nM) produced additive transcriptional response in a concentration-dependent manner (Chen et al., 1997). The authors concluded that co-exposure of DDT isomers and metabolites with E_2 or another DDT isomer and metabolite can have an additive effect on hER transcriptional activation.

The present study (**Fig. 3.6**) is also in agreement with the work of Chen et al. (1997) in which *p,p′*-DDT was able to activate hER response at 1 µM and induced a maximum response of 49.5% in yeast-based reporter assay whereas *p,p′*-DDE had no effect (Chen et al., 1997). However, we observed that *p,p′*-DDT and *p,p′*-DDE were able to cause transcriptional activation of the hER, with varying responses. There are other differences that can be observed between the study mentioned above (Chen et al., 1997) and this current study. One major difference is that Chen and colleagues Chen et al., 1997) used a yeast-based reporter gene assay, instead of the mammalian-based reporter gene assay applied in our study. The yeast-based RGAs have been shown to be less sensitive compared to the mammalian-based cellular reporter systems. For instance, the concentration of E2 that induced maximal hER transcriptional response was 10-fold higher than the dose eliciting maximal hER transcriptional response in this study (**Fig. 3.6; Table 3.1**). In addition, Chen et al. (1997) only evaluated effects of different

concentration of *p,p′*-DDT (40 nM, 100 nM and 1000 nM) on the hER transcriptional response mediated by 0.3 nM of E_2. In contrast, our study used mammalian-based RGA (MMV-Luc) to evaluate the effect of *p,p′*-DDT and *p,p′*-DDE, either in single or binary mixtures, on the hER transcriptional activation elicited by two doses of E2 (0.05 nM and 10 nM). This current study (Fig. 3.7 - 3.9) also determined the effect of *p,p′*-DDT or *p,p′*-DDE in combination with either ZEN or α-ZOL, on E2-mediated hER transcriptional response.

3.3.4 Interactive effects of mixtures of mycotoxins and pesticides on E_2-mediated ER transcriptional activation

Since human beings and animals are not exposed to food and environmental oestrogenic chemicals alone, but in mixtures with other oestrogenic chemicals, this study also examined the effect of such mixtures on E_2-induced hER transcriptional activity. It was observed that mixtures of ZEN and α-ZOL, ZEN and *p,p′*-DDT, ZEN and *p,p′*-DDE, α-ZOL and *p,p′*-DDT, and α-ZOL and *p,p′*-DDE when co-treated with E_2 (0.05 nM) enhanced E_2-induced hER transcription at low dose combinations, but had additive or antagonistic effects at high dose combinations (**Fig. 3.8 & 3.9**). However, the above mixtures caused dose-dependent reduction (antagonistic effects) on E_2-induced hER transcription when co-exposed with 10 nM of E_2. It has also been demonstrated that co-treatment of low concentrations of oestrogen agonists 4-Nonylphenol, *o,p′*-DDT, methoxychlor, chlordane, endosulfan and dieldrin at 200 nM to 2 µM with low dose of E_2 (0.003 nM) in T47D-ER CALUX assay caused induction of transcriptional response which were significantly different from the response of the individual treatments (Legler et al., 1999), similar to the result of this study. These results indicate that oestrogenic chemicals may act synergistically or antagonistically depending on combination ratio or concentration and further research is warranted in the *in vivo* effects of mixtures of xeno-oestrogens.

A major observation of this study is that exposure of equimolar concentrations of *p,p′*-DDT and *p,p′*-DDE mixtures with either 0.05 nM or 10 nM of E_2 significantly reduced transcriptional response in concentration-dependent fashion and this response was completely inhibited at equimolar combinations (50 µM) with both 0.05 nM and 10 nM

of E_2. This observation was not as a result of cell cytotoxicity as there was either a significant increase in cell number or no effect on cell viability at these concentrations (**Fig. 3.3**). In agreement with this study, the β-galactosidase activity was completely abolished when *p,p′*-DDT (100 µM) and *p,p′*-DDE (100 µM) were combined with 0.2 nM of E_2 in a yeast-based hER reporter gene assay, but significantly increased progesterone-mediated PR transcriptional activation (Li et al., 2008). Interestingly, serum *p,p′*-DDT and *p,p′*-DDE concentrations of 40.311 µM and 86.155 µM, respectively have been reported in human epidemiological studies (Dua et al., 2001; Longnecker et al., 2002) indicating clinical relevance of the effects observed in this study.

3.4 Conclusion

In conclusion, this study shows that ZEN and *p,p′*-DDT as well as their major metabolites are ER agonists and the results obtained using the MMV-Luc reporter gene assay are similar to other mammalian-based reporter gene assay cell lines. In addition, this study has highlighted that ZEN and α-ZOL have biphasic effects on E_2-mediated ER transcriptional response depending on the concentration of E_2 present as well as concentration of each compound during co-exposure. Interestingly, mixtures equimolar concentrations of *p,p′*-DDT and *p,p′*-DDE at 50 µM completely inhibited the response induced by either 0.05 nM or 10 nM of E_2. It is important to assess the interactive effects of oestrogenic compounds either in single or mixtures with concentrations of E_2 relevant to human exposure during risk assessment using ER transactivation assays to understand their effects on human reproductive health. The mechanisms through which mycotoxins and persistent organochloride pesticides cause reproductive toxicity are not currently known in detail, but it is most likely that these compounds act through different mechanisms. Therefore, it becomes pertinent that different *in vitro* methods such as hormone and gene expression assays should be used in further research to understand the mechanistic impacts of different mycotoxins and persistent organochloride pesticides on reproduction.

Chapter 4

Analysis of individual and combined effects of mycotoxins and persistent organochloride pesticides on the viability and steroid hormone production in MA-10 murine Leydig cell line

4.1 Introduction

Epidemiological studies have shown strong deterioration in male reproductive health globally (Kumar et al., 2015; Sengupta et al., 2017). A study examining the sperm concentration of 14,947 fertile men between 1940 and 1990 reported a 50% decline in sperm concentration and increased abnormalities in sperm morphology and motility worldwide (Carsen et al., 1992). Several other reports support the continuous decline in male fertility as a result of the increase in poor sperm quality globally (Swan et al., 1997, 2000). In a more recent and robust systematic review and meta-regression analysis, Levine et al. (2017) reported a significant reduction (50 - 60%) in both sperm concentration and total sperm count among men from North America, Europe, Australia and New Zealand from between 1973 and 2011. It is estimated that in 20% of infertility cases, the pathology is solely from the man but both male and female factors also contribute 30% - 40% (Phillips and Tanphaichitr, 2008). Therefore, the male factor is at least partly responsible for infertility in about 50% of cases. The causes of male infertility are multi-factorial and these are still poorly understood. Male infertility may be due to several conditions, including congenital maldescent of the testis, testicular varicocele, untreated or poorly managed sexually transmitted infections, previous genital tract surgery, and hormonal abnormalities (Keck et al., 1998; Ozoemena et al., 2011; Jimoh et al., 2012). Microdeletions in the azoospermia factor (AZF) regions (AZFa, AZFb and AZFc) of the long arm of Y chromosome has also been associated with teratozoospermia and oligospermia, and affects about 3 - 13% of infertile men (Mascarenhas et al., 2016). In infertile males, sperm DNA damage are also common and may be caused by exposure to environmental chemicals (Povey and Stocks, 2010; Stocks et al., 2010; Pacey, 2010). Several reports have shown that there is a general decline in male fertility mainly due to poor sperm quality (Carlsen et al, 1992; Swan et al, 1997, 2000). The global decline in male fertility and the occurrence of testicular dysgenesis syndrome can be attributed to exposure to endocrine disrupting chemicals

(EDCs) in the environment, food and pharmaceutical products, including mycotoxins and pesticides (Ibe et al., 1994; Uriah et al., 2001; Martenies and Perry, 2013; Main et al., 2010; Eze and Okonofua, 2015; Kumar et al., 2015; Chiu et al., 2016; Skakkebaek et al., 2016).

Exposure to various mycotoxins and pesticides is common and can be high in some populations, especially in developing countries. Aflatoxins (AFs), fumonisins (FUMs), ochratoxin A (OTA), deoxynivalenol (DON) and zearalenone (ZEN) are considered to be the major mycotoxins of public health concern due to their frequent occurrence in cereals and cereal-based products. Whilst there is evidence of health effects of these different mycotoxins (Rodrigues and Naehrer, 2012; Wild and Gong, 2010), there have been few studies of the role of mycotoxins on adverse reproductive health in human populations. AFB_1 in serum (4.8 µM) and semen (5.3 µM) have been linked to abnormalities in sperm count, morphology and motility among infertile males in Nigeria (Ibe et al., 1994; Uriah et al., 2001). Studies have reported high levels of exposure to OTA (Steyn, 1993; Maaroufi et al., 1995) and/or DON (Sarkanj et al., 2013; Gong et al, 2015; Wells et al., 2016), but there is currently no data on the potential role of these mycotoxins in reproductive health.

ZEN is known to cause adverse reproductive effects in farm animals (Zinedine et al., 2007) and as ZEN and metabolites are known to bind to oestrogen receptors disrupting the binding of 17β-oestradiol (Kuipers et al., 1998), there has been some interest in possible effects on human reproductive health. Mean concentrations of ZEN (167.0±17.1 ng/mL; 0.525±0.054 µM) were detected in endometrial tissues of patients with endometrial hyperplasia (Tomaszewski et al., 1998) while concentration of ZEN (200 – 475 ng/mL; 0.628 - 1.492 µM) have been reported in individuals with breast and cervical cancer (Pillay et al., 2002). A concentration of ZEN at 18.9 - 103 ng/mL (0.059 - 0.324 µM) was associated with early thelarche/mastopathy in young girls (Szuets et al., 1997) while ZEN (0.7235 – 1.1439 ng/mL; 2.0 - 4.0 nM) and α-ZOL (0.1045 - 0.1085 ng/mL; 0.033 - 0.034 nM) were linked to precocious puberty in young girls from North-West Tuscany, Italy (Massart et al., 2008, 2010). Similar concentrations of ZEN as found in human blood (150 ng/mL; 0.471 µM) caused reduction in sperm

concentration and increase in sperm abnormalities in male mice (Zatecka et al., 2014) while ZEN concentration as low as 30 ng/mL (0.094 µM) caused sperm DNA instability in boars (Tsakmakidis et al., 2008).

In sub-Saharan Africa, in addition to mycotoxin exposure, exposure to organochloride pesticides is also common due to poor regulation of their use (Bouwman et al., 2011). The organochloride pesticide 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane (*p,p'*-DDT) is a pesticide/insecticide previously used globally in the control of insects on agricultural crops as well as in mitigating insect vectors of diseases (ATSDR, 2002). Its metabolite, 1,1-dichloro-2,2-bis(p-chlorophenyl) ethylene (*p,p'*-DDE) is found in both the biological systems and the environment, and has a longer half-life compared to *p,p'*-DDT (ATSDR, 2002). In humans, *p,p'*-DDE levels of 232 ng/mL (0.729 µM) have been linked to reduction in testosterone levels and free androgen index (Martin et al., 2002). In agreement with the above study, Ayotte et al. (2001) reported that a mean serum *p,p'*-DDE of 600 ng/mL (1.887 µM) in Mexican men decreased serum and bioavailable testosterone, decreased semen ejaculation volume and reduced sperm count. In subsequent studies, *p,p'*-DDT and *p,p'*-DDE had adverse effects on human sperm motility, morphology, count, and semen volume (De Jager et al., 2006; Aneck-Hahn et al., 2007), and reduced the production of reproductive hormones, especially testosterone (Giwercman et al., 2006; Asawasinsopon et al., 2006). Furthermore, an increased risk of birth defects, including reduced anogenital distance, cryptorchidism and urogenital malformation has also been associated with *p,p'*-DDT (Salazar-Garcia et al., 2004; Bornman et al., 2010) and *p,p'*-DDE (Longnecker et al., 2002).

The Leydig cells in the males' testes are responsible for producing androgens (testosterone and dihydrotestosterone) and these steroid hormones play major roles in male sexual differentiation (during embryogenesis), pubertal development, spermatogenesis and other reproductive function (Akingbemi, 2005). However, reports also indicate that oestrogen is involved in spermatogenesis through the stimulation of germ cell proliferation, differentiation and final maturation of spermatids as well as germ cell survival (Carreau and Hess, 2010). Alteration in steroidogenesis has been linked to poor reproductive function and developmental defects in males (Yeung et al.,

2011). Most studies investigating the effects of chemicals interfering with steroid hormone biosynthesis have applied the H295R human adrenocortical carcinoma cell line for the assessment of adrenal production of 17β-oestradiol and testosterone (Hecker et al., 2006, 2011; Pinto et al., 2018). It has been argued that the H295R steroidogenesis assay identifies only alterations in adrenal hormone production, and may not accurately determine perturbations in testicular steroidogenesis (Strajhar et al., 2017). This is due to low levels of testosterone produced by H295R cells even though it contains all the adrenal steroidogenic enzymes and also, the Nu-serum in H295R cells growth media contribute substantial concentration of progesterone and testosterone which may give false increase in progesterone and testosterone levels in the assay (Zhang et al., 2011; Nakano et al., 2016; Strajhar et al., 2017). This is true as seventeen (17) out of twenty (20) chemicals (85%) which did not reduce testosterone level in the H295R steroidogenesis assay were found to inhibit testosterone production in Leydig cells (Principato et al., 2018). In addition, H295R cell line lacks functional luteinising hormone (LH) and follicle-stimulating hormone (FSH) receptors, and chemicals that alter steroidogenesis by affecting gonadotropin receptors may not be detected by the H295R assay (Pinto et al., 2018). Therefore, the Leydig cell assay is recommended to complement the H295R assay in elucidating the effects of chemicals that alter testicular steroidogenesis (Pinto et al., 2018; Principato et al., 2018).

The MA-10 Leydig cell line was chosen for this study as it has been recommended as a useful model for assessing Leydig cell function and impacts of environmental toxins on the Leydig cells due to its ability to produce progesterone and testosterone, and express mRNA for steroidogenic enzymes, including steroid acute regulatory protein (StAR), 17α-hydroxylase/17,20-lyase type 1 (Cyp17a1), cytochrome P450 cholesterol side-chain cleavage enzyme (Cyp11a1), 3β-hydroxysteroid dehydrogenase type 1 (3β-hsd1) and 17β-hydroxysteroid dehydrogenase type 3 (17β-hsd3) (Clewell et al., 2010; Dankers et al., 2013; Roelofs et al., 2014, 2015). Human beings are likely to be exposed to several mycotoxins concurrently through food intake since most fungi can produce multiple mycotoxins simultaneously, and therefore foods can be contaminated with various mycotoxins at the same time (Streit et al., 2012; Li et al., 2014a). It becomes pertinent to assess the toxicity of mycotoxin mixtures on human reproductive health. In addition, co-exposures of OTA, DON, ZEN, α-ZOL and β-ZOL or *p,p'*-DDT is common in many

developing countries, so this study has investigated the effects of such co-exposures or cocktails as well as single exposures on the cell viability and steroid hormone production of Leydig cells *in vitro*, at doses consistent with concentrations that have been reported in human exposure studies (Frizzel et al., 2011, Clarke et al., 2014; Dua et al., 2001; Abid-Essefi et al., 2004**;** Bensassi et al., 2009, 2014).

4.2. Results

4.2.1. Effects of individual mycotoxins and pesticides on cell viability

With single toxin treatment at doses between 0.1 to 64 µM for 48 hours, DON was the most cytotoxic to MA-10 Leydig cells (IC_{50}: 21.97 ± 5.25 µM), reducing the cell viability in dose dependent manner compared to 0.1% DMSO vehicle control (**Fig. 4.1D**). OTA was the second most cytotoxic to MA-10 cells with an IC_{50} of 28.50 ± 2.63 µM followed by α-ZOL (IC_{50}: 30.10 ± 0.19 µM), and ZEN (IC_{50}: 60.10 ± 3.59 µM). ZEN, α-ZOL and β-ZOL significantly stimulated the proliferation of MA-10 cells ($P \leq 0.01$) at 16 µM (112.0%, 117.0% and 127.0%, respectively) with all the 3 toxins causing strong toxicity at 64 µM, decreasing the cell viability to 18.0%, 11.0% and 47.0%, respectively (**Figs. 4.1F – 4.1H**). *p,p′*-DDT showed slight toxicity between 0.1 and 32 µM, and significantly reduced cell viability to 56.0% at 64 µM ($P \leq 0.0001$). However, *p,p′*-DDE and AFB_1 only showed low toxicity to the MA-10 cells, even at the highest dose tested (64 µM) when compared to 0.1% DMSO control (**Figs. 4.1A & 4.1B**).

4.2.2. Effects of binary combinations of *p,p′*-DDT or *p,p′*-DDE with mycotoxins on MA-10 Leydig cell viability

Neither *p,p′*-DDT nor *p,p′*-DDE induced significant cytotoxicity when the cells were treated with the compound individually, except that at 64 µM *p,p′*-DDT induced approximately 40% cell death (**Fig. 4.1A**). Cytotoxicity at this dose combination (64 µM) was enhanced when cells were treated with *p,p′*-DDT and *p,p′*-DDE together (about 95% cell death, **Fig. 4.3**). For all other compounds tested, co-treatment with *p,p′*-DDT enhanced toxicity, whereas co-treatment with *p,p′*-DDE did not, except at high doses. For example, whereas low cytotoxicity was seen for *p,p′*-DDT or AFB_1 individually below 64 µM (**Figs. 4.1A & 4.1C**), a dose response was seen for *p,p′*-DDT

+ AFB_1, with significant cytotoxicity at 16 µM ($P \leq 0.0001$; **Fig. 4.2A**). In contrast, the cytotoxicity of the various mycotoxins was not altered by the addition of *p,p'*-DDE and in cases where it occurred, it was only at the highest doses tested (**Fig 4.3**).

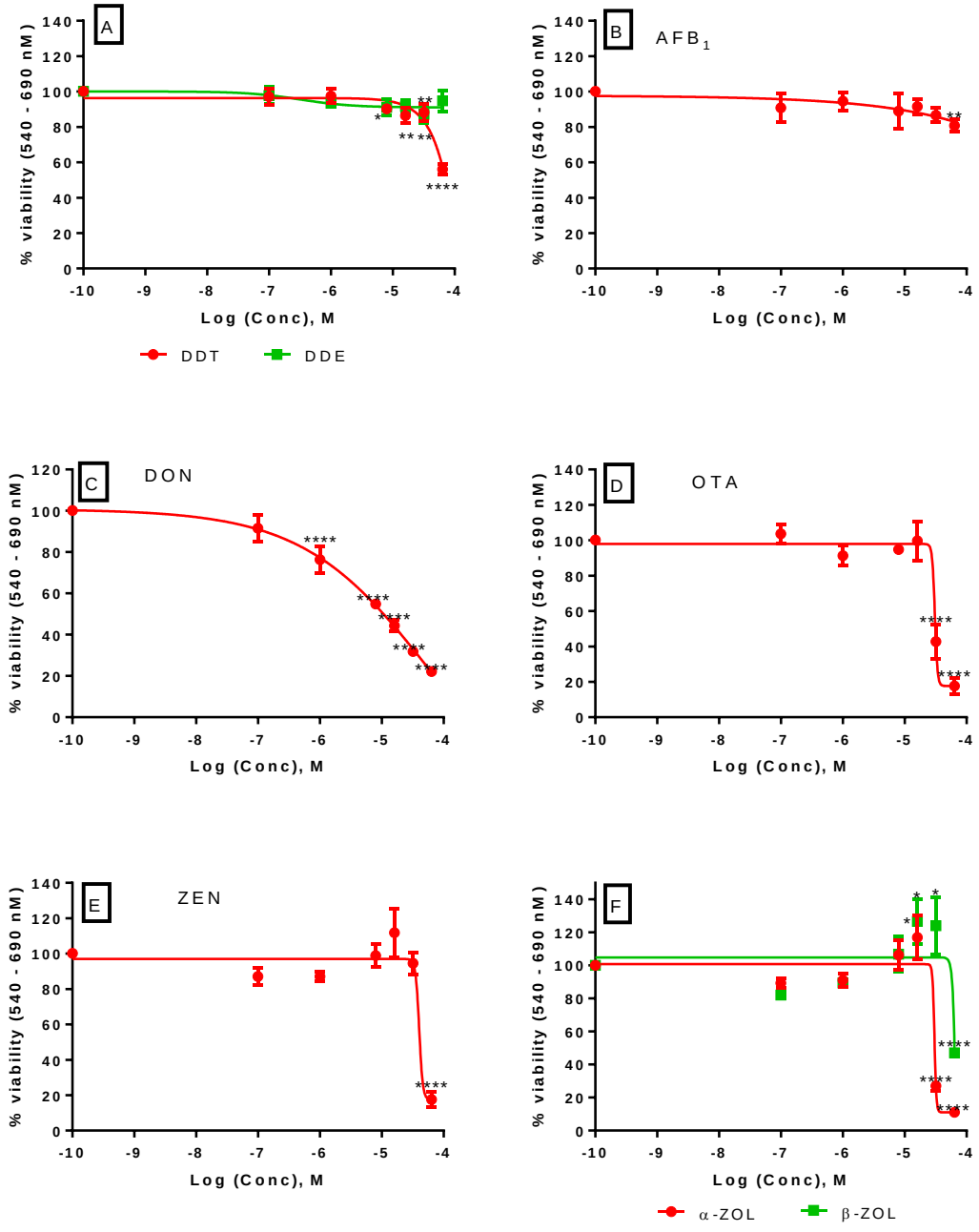

Fig. 4.1: Dose-response curve of the cell viability results using MTT for MA-10 Leydig cells exposed to graded concentrations of mycotoxins and pesticides for 48 hours calculated as a percentage of 0.1% DMSO vehicle control. Each value on the graph is the mean of three independent experiments with three technical replicates each and error bars show the standard deviation (SD) of three biological replicates. Data were analysed using one-way ANOVA with Dunnett's test method to correct for multiple comparison.

$p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****) represent significant effects.

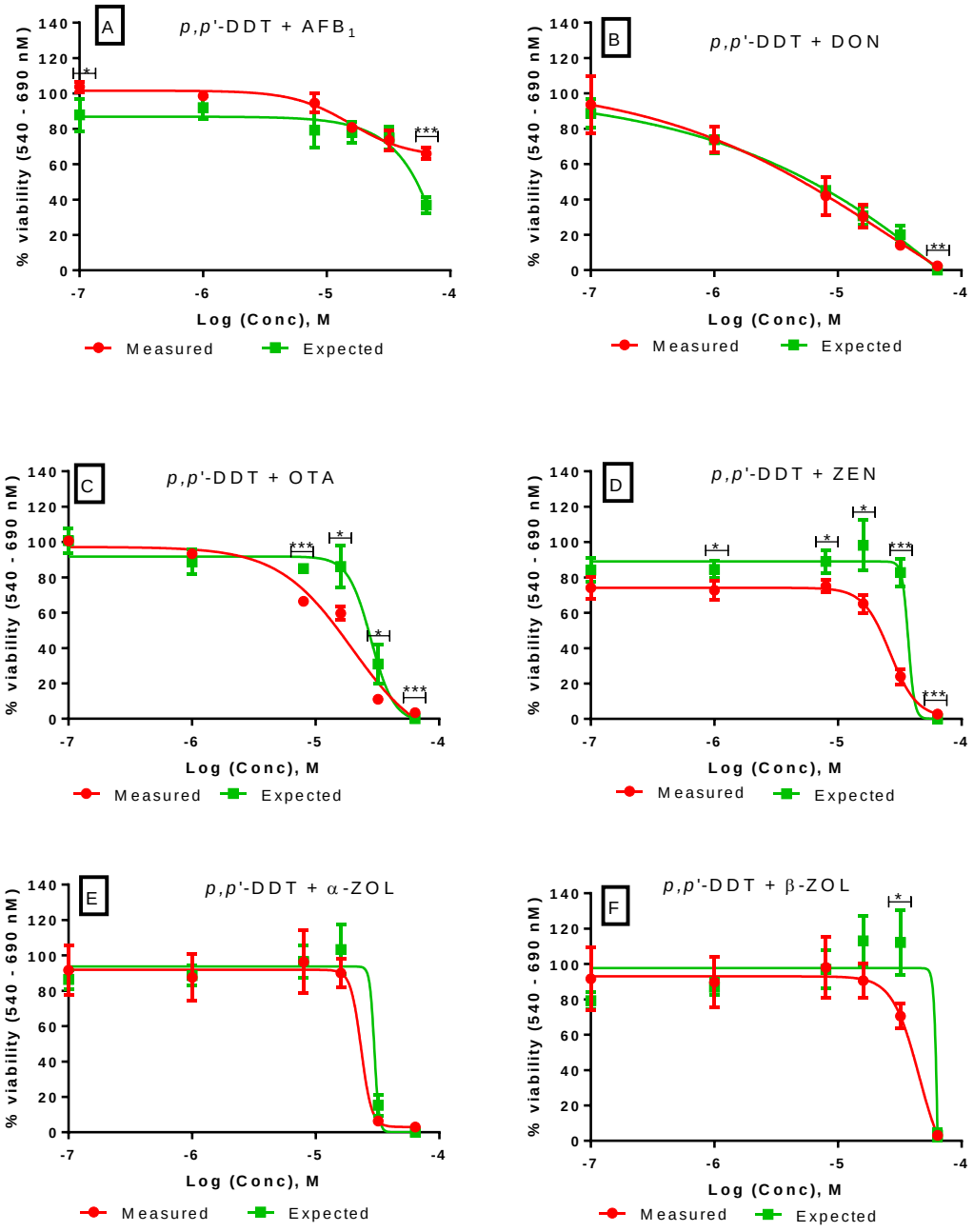

Fig. 4.2: Measured and expected cell viability results using MTT for MA-10 Leydig cells exposed to chemically-defined binary combinations of *p,p'*-DDT and mycotoxins for 48 hours calculated as a percentage of 0.2% DMSO vehicle control. Each value on the graph is the mean of three independent experiments with three technical replicates each and error bars show the standard deviation (SD) of three biological replicates. Data were analysed using multiple t-test with Holm-Šídák test method to correct for multiple

comparison. $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****) represent significant deviation from additive effects.

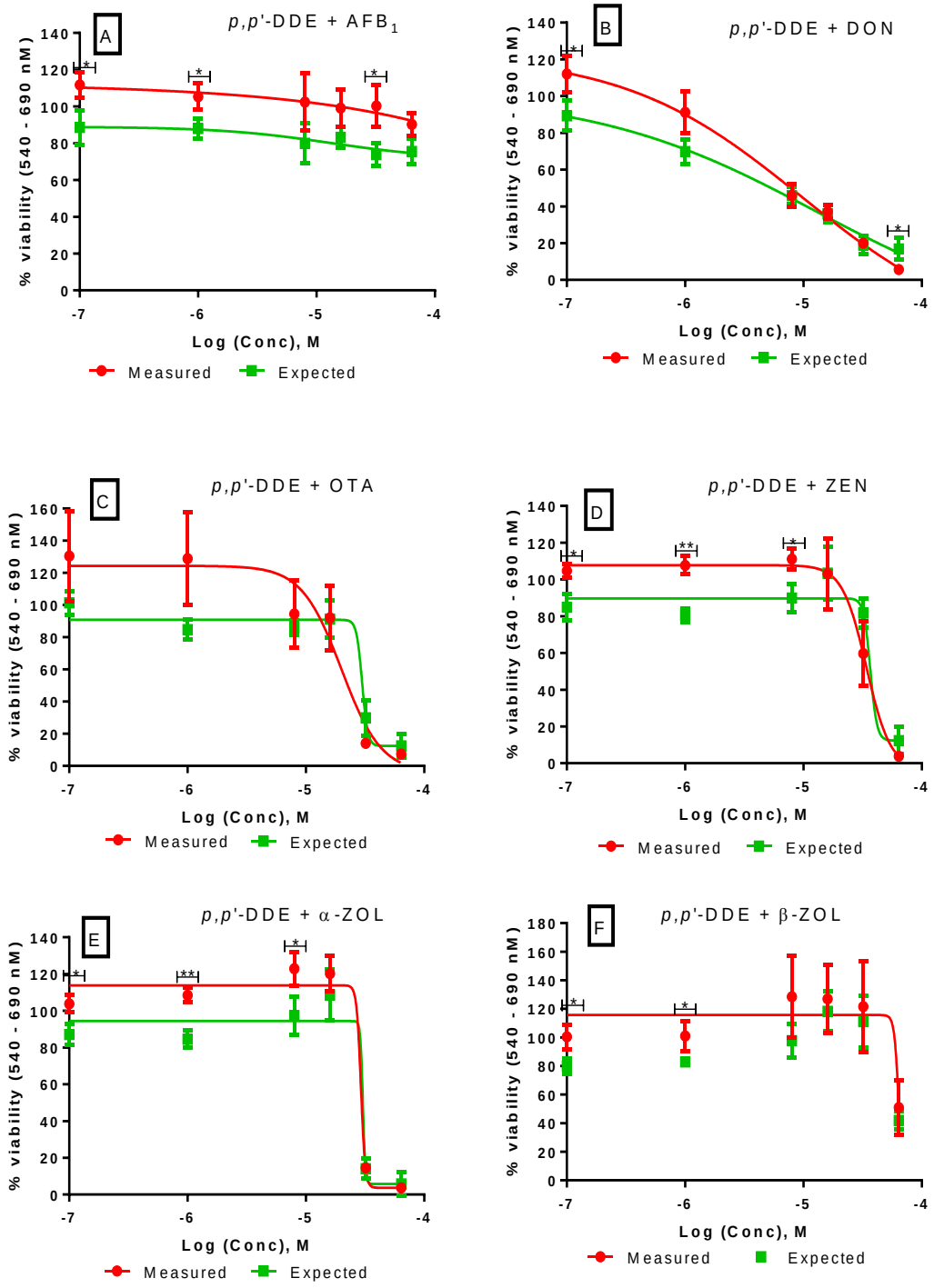

Fig. 4.3: Measured and expected cell viability results using MTT for MA-10 Leydig cells exposed to chemically-defined binary combinations of *p,p'*-DDE and mycotoxins for 48 hours calculated as a percentage of 0.2% DMSO vehicle control. Each value on the graph is the mean of three independent experiments with three technical replicates

each and error bars show the standard deviation (SD) of three biological replicates. Data were analysed using multiple t-test with Holm-Šídák test method to correct for multiple comparison. $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****) represent significant deviation from additive effects.

4.2.3 Effects of binary mycotoxin mixtures on MA-10 Leydig cell viability

OTA did not induce much cytotoxicity up to 16 µM when cells were treated with only this compound, but DON/OTA toxicity was increased at 8 µM - 32 µM compared to DON alone (**Fig. 4.4**A). OTA also had a synergistic effect on toxicity induced by ZEN, α-ZOL and β-ZOL (**Fig. 4.5A**). There was no increase in cytotoxicity seen for co-treatment with OTA and AFB_1. Co-treatment with none of the other mycotoxins had a noticeable impact on the cytotoxicity of ZEN, α-ZOL or β-ZOL. Interestingly, AFB_1, which showed no toxicity up to 32 µM, reduced the toxicity due to DON when cells were treated with both mycotoxins at the same time (**Fig. 4.3A**). Such a reduction in toxicity were not seen when cells were treated with AFB_1 together with OTA.

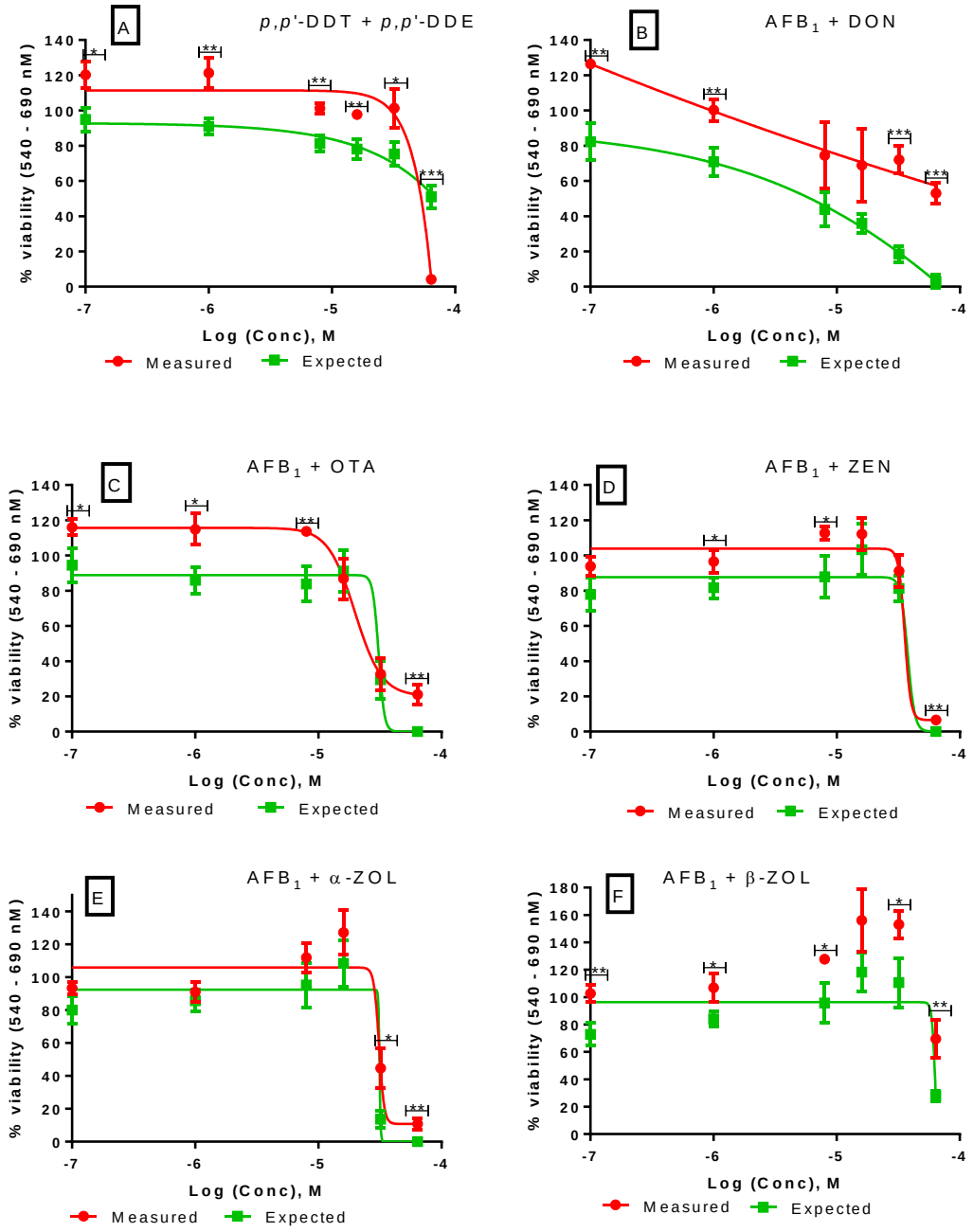

Fig. 4.4: Measured and expected cell viability results using MTT for MA-10 Leydig cells exposed to chemically-defined binary combinations of *p,p'*-DDT and *p,p'*-DDE metabolite (A) and AFB_1 with other mycotoxins (B – F) for 48 hours calculated as a percentage of 0.2% DMSO vehicle control. Each value on the graph is the mean of three independent experiments with three technical replicates each and error bars show the standard deviation (SD) of three biological replicates. Data were analysed using multiple t-test with Holm-Šídák test method to correct for multiple comparison. $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****) represent significant deviation from additive effects.

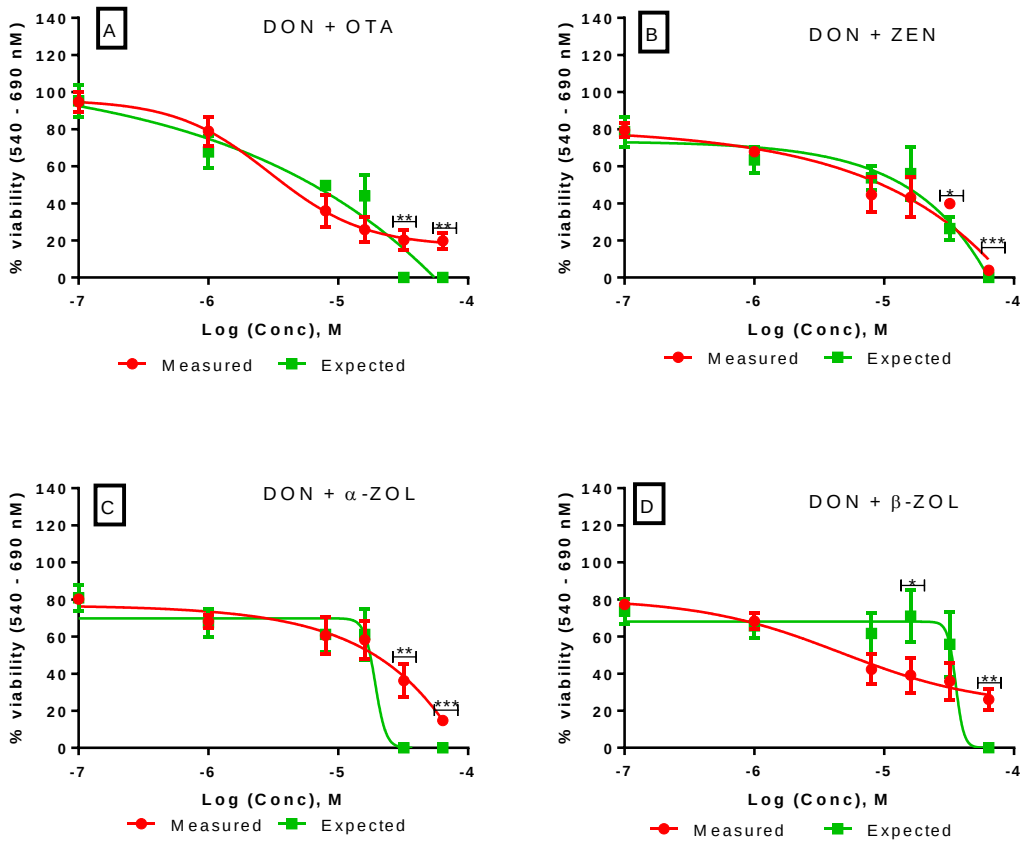

Fig. 4.5: Measured and expected cell viability results using MTT for MA-10 Leydig cells exposed to chemically-defined binary combinations of DON and OTA, ZEN, α-ZOL and β-ZOL for 48 hours calculated as a percentage of 0.2% DMSO vehicle control. Each value on the graph is the mean of three independent experiments with three technical replicates each and error bars show the standard deviation (SD) of three biological replicates. Data were analysed using multiple t-test with Holm-Šídák test method to correct for multiple comparison. $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****) represent significant deviation from additive effects.

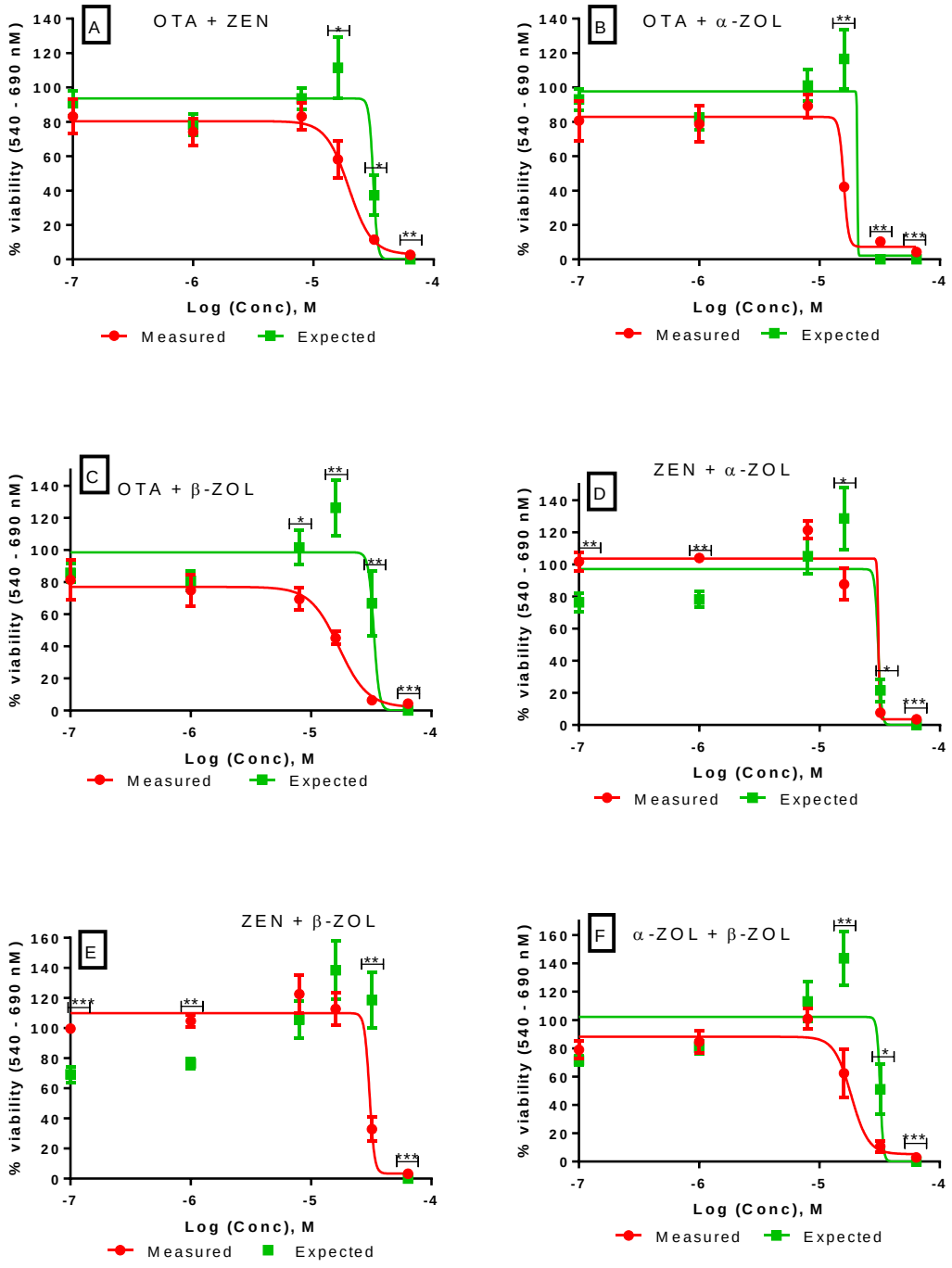

Fig. 4.6: Measured and expected cell viability results using MTT for MA-10 Leydig cells exposed to chemically-defined binary combinations of OTA with either ZEN, α-ZOL or β-ZOL (A - C), and ZEN with its metabolites (D - F) for 48 hours calculated as a percentage of 0.2% DMSO vehicle control. Each value on the graph is the mean of three independent experiments with three technical replicates each and error bars show the standard deviation (SD) of three biological replicates. Data were analysed using multiple t-test with Holm-Šídák test method to correct for multiple comparison. $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****) represent significant deviation from additive effects.

4.2.4 Interactive effects of binary mycotoxin and pesticide mixtures on MA-10 Leydig cell viability

It was observed that the combinatory effects of the mycotoxin (ZEN) and pesticide (*p,p'*-DDT) on MA-10 Leydig cells was synergistic in nature for all the doses tested (**Fig. 4.2D**), except at 64 µM when there was an exhibition of antagonistic effect (**Table 4.1**). In addition, combinations of DON/OTA, DON/ZEN, DON/α-ZOL, DON/β-ZOL, OTA/ZEN, OTA/α-ZOL, and OTA/β-ZOL were generally additive and synergistic at low concentrations, but antagonistic at 64 µM combinations (**Fig. 4.5 & Fig. 4.6**). In contrast, combinations of *p,p'*-DDE with all the mycotoxins tested showed antagonistic effects at lower concentrations (1 – 8 µM) but showed additive effects at concentrations ranging from 16 µM to 64 µM combinations (**Fig. 4.3; Table 4.1**). In general, most of the mycotoxins and pesticides combinations showed antagonistic effects at high doses (**Table 4.1**).

Table 4.1: Interactive effects of binary mixtures of mycotoxins and pesticides in MA-10 cell line

Toxin combinations	**Dose combinations (µM) and interaction effects of binary mixtures**					
	0.1: 0.1	**1 : 1**	**8 : 8**	**16 : 16**	**32 : 32**	**64 : 64**
p,p′-DDT + *p,p′*-DDE	p = 0.01308[b]	p = 0.005931[b]	p = 0.003594[b]	p = 0.005382[b]	p = 0.02532[b]	p = 0.0002399[a]
	Diff. = 25.33	Diff. = 30.33	Diff. = 20.00	Diff. = 19.56	Diff. = 26.00	Diff. = -46.78
p,p′-DDT + AFB1	p = 0.04732[b]					p = 0.000807[b]
	Diff. = 15.8878					Diff. = 29.11
p,p′-DDT + DON						p = 0.001143[b]
						Diff. = 2.44
p,p′-DDT + OTA			p = 0.0002238[a]	p = 0.02106[a]	p = 0.03546[a]	p = 0.0003035[b]
			Diff. = 18.56	Diff. = 26.44	Diff. = 20.00	Diff. = 3.44
p,p′-DDT + ZEN		p = 0.49107[a]	p = 0.02999[a]	p = 0.02019[a]	p = 0.0003590[a]	p = 0.0002356[b]
		Diff. = 11.78	Dif. = 13.99	Diff. = -33.11	Diff. = -58.78	Diff. = 2.778
p,p′-DDT + β-ZOL					p = 0.0208[a]	
					Diff. = -41.664	

Toxin combinations	Dose combinations (µM) and interaction effects of binary mixtures					
	0.1: 0.1	**1 : 1**	**8 : 8**	**16 : 16**	**32 : 32**	**64 : 64**
p,p′-DDE + AFB_1	p = 0.02687[b]	p = 0.03065[b]			p = 0.02462[b]	
	Diff. = 23.11	Diff. = 17.33			Diff. = 26.44	
p,p′-DDE + DON	p = 0.03698[b]					p = 0.03530[b]
	Diff. = 22.78					Diff. = -11.22
p,p′-DDE + ZEN	p = 0.01266[b]	p = 0.001662[b]	p = 0.01858[b]			
	Diff. = 19.78	Diff. = 27.11	Diff. = 21.22			
p,p′-DDE + α-ZOL	p = 0.01807[b]	p = 0.002741[b]	p = 0.03204[b]			
	Diff. = 16.67	Diff. = 23.89	Diff. = 25.56			
p,p′-DDE + β-ZOL	p = 0.02514[b]	p = 0.047042[b]				
	Diff. = 20.55	Diff. = 18.11				
AFB_1 + DON	p = 0.001962[b]	p = 0.07659[b]			p = 0.0005173[b]	p = 0.0002258[b]
	Diff. = 44.11	Diff. = 29.33			Diff. = 53.50	Diff. = 50.11
AFB_1 + OTA	p = 0.02486[b]	p = 0.01254[b]			p = 0.007189[b]	p = 0.003325[b]
	Diff. = 21.56	Diff. = 29.00			Diff. = 29.89	Diff. = 21.00

Toxin combinations	Dose combinations (µM) and interaction effects of binary mixtures					
	0.1: 0.1	**1 : 1**	**8 : 8**	**16 : 16**	**32 : 32**	**64 : 64**
AFB_1 + ZEN	p = 0.04086[b]	p = 0.02449[b]				p = 0.001646[b]
	Diff. = 14.89	Diff. = 24.89				Diff. = 6.56
AFB_1 + α-ZOL					p = 0.01505[b]	p = 0.006154[b]
					Diff. = 31.00	Diff. = 10.78
AFB_1 + β-ZOL	p = 0.007783[b]	p = 0.02868[b]	p = 0.02003[b]		p = 0.02408[b]	p = 0.007889[b]
	Diff. = 29.78	Diff. = 22.89	Diff. = 32.00		Diff. = 42.33	Diff. = 41.67
DON + OTA					p = 0.003077[b]	p = 0.001194[b]
					Diff. = 20.22	Diff. = 19.78
DON + ZEN					p = 0.0495[b]	p = 0.000940[b]
					Diff. = 13.50	Diff. = 3.89
DON + α-ZOL					p = 0.002155[b]	p = 0.002188[b]
					Diff. = 36.22	Diff. = 14.78
DON + β -ZOL				p = 0.02976[a]		p = 0.001483[b]
				Diff. = -31.89		Diff. = 26.11

Toxin combinations	Dose combinations (µM) and interaction effects of binary mixtures					
	0.1: 0.1	**1 : 1**	**8 : 8**	**16 : 16**	**32 : 32**	**64 : 64**
OTA + ZEN				p = 0.01116[a]	p = 0.01800[a]	p = 0.001324[b]
				Diff. = -53.33	Diff. = -25.89	Diff. = 2.67
OTA + α-ZOL				p = 0.001818[a]	p = 0.01800[b]	p = 0.006852[b]
				Diff. = -74.33	Diff. = 10.33	Diff. = 4.22
OTA + β -ZOL			p = 0.01211[a]	p = 0.001441[a]	p = 0.006446[a]	p = 0.0002020[b]
			Diff. = -32.12	Diff. = -81.11	Diff. = -60.33	Diff. = 4.33
ZEN + α-ZOL	p = 0.005475[b]	p = 0.001045[b]		p = 0.03032[a]	p = 0.02633[a]	p = 0.0002681[b]
	Diff. = 25.34	Diff. = 25.78		Diff. = -41.00	Diff. = -13.89	Diff. = 3.55
ZEN + β -ZOL	p = 0.0008886[b]	p = 0.000902[b]			p = 0.001830[a]	p = 0.0005620[b]
	Diff. = 30.45	Diff. = 28.22			Diff. = -85.67	Diff. = 3.33
α-ZOL+ β -ZOL				p = 0.005404[a]	p = 0.01823[a]	p = 0.0006013 [b]
				Diff. = -81.11	Diff. = -40.33	Diff. = 2.89

Keys. p: p-value; **Diff.:** mean difference**; [a]Synergistic:** measured cell viability values were significantly below expected values; **[b]Antagonistic:** measured cell viability values were significantly above expected values. $AFB_{1:}$ aflatoxin B_1; OTA: ochratoxin A; DON: deoxynivalenol; ZEN: zearalenone; α-ZOL: alpha-zearalenol; β-ZOL: beta-zearalenol; *p,p'*-DDT: 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane and *p,p'*-DDE: 1,1-dichloro-2,2-bis(p-chlorophenyl) ethylene

4.2.5 Effects of single mycotoxins and pesticide on progesterone secretion

The basal P4 level in the residual cell culture medium of MA-10 cells treated with DMSO (0.1%) for 48 h was 0.60 ± 0.07 ng/mL. After treatment with the positive control (8-Br-cAMP: 50 μM) for 48 h, the concentration of P4 secreted by the MA-10 cells was increased to 135.50 ± 44.34 ng/mL (680-fold). Generally, the maximum concentration of P4 occurred at 16 μM for *p,p'*-DDT, ZEN, β-ZOL, DON and OTA whereas peak P4 level was observed at 0.1 μM in α-ZOL with the lowest P4 concentration at 16 μM (**Fig. 4.7**). Among the tested compounds, the highest dose of ZEN employed (16 μM) induced the maximum P4 level in MA-10 cells followed by OTA at 16 μM (**Fig. 4.7B and 4.7F**). P4 production following ZEN exposure for 48 h was significantly increased in all the doses tested compared to the solvent control ($P \leq 0.0001$) with the highest P4 secretion occurring at 16 μM (4.7 ± 0.48 ng/mL). At 8 and 16 μM of OTA, the level of P4 in MA-10 cells was significantly increased ($P \leq 0.0001$) with the highest concentration occurring at 16 μM of OTA (3.7 ± 0.6 ng/mL). The lowest concentrations of *p,p'*-DDT (0.1 - 8 μM) had no effect on P4 release in MA-10 cells, but *p,p'*-DDT (16 μM) significantly ($p \leq 0.001$) induced the level dose-dependently (**Fig. 4.8A**).

4.2.6 Effects single mycotoxins and pesticide on testosterone production

The basal testostosterone level in the residual cell culture medium of MA-10 cells treated with DMSO (0.1%) was 251.6 ± 20.7 pg/mL after 48 h exposure. Exposure of MA-10 cells with the (8Br-cAMP: 50 μM) positive control induced testostosterone level by 21-fold (5233.9 ± 594.63 pg/mL) after 48 h. There was a reduction of testostosterone level after *p,p'*-DDT (8 and 16 μM) treatment, but this was not significantly different from the vehicle control (**Fig. 4.8A**). Generally, ZEN, α-ZOL, β-ZOL and DON at 8 and 16 μM strongly inhibited testostosterone production in exposed cells ($p \leq 0.01$; **Fig. 4.8B – 4.8E**). Following OTA treatment at 0.1, 1 and 8 μM, the level of testostosterone increased by approximately 2-fold compared to the vehicle control but this was no significantly different (**Fig. 4.8F**).

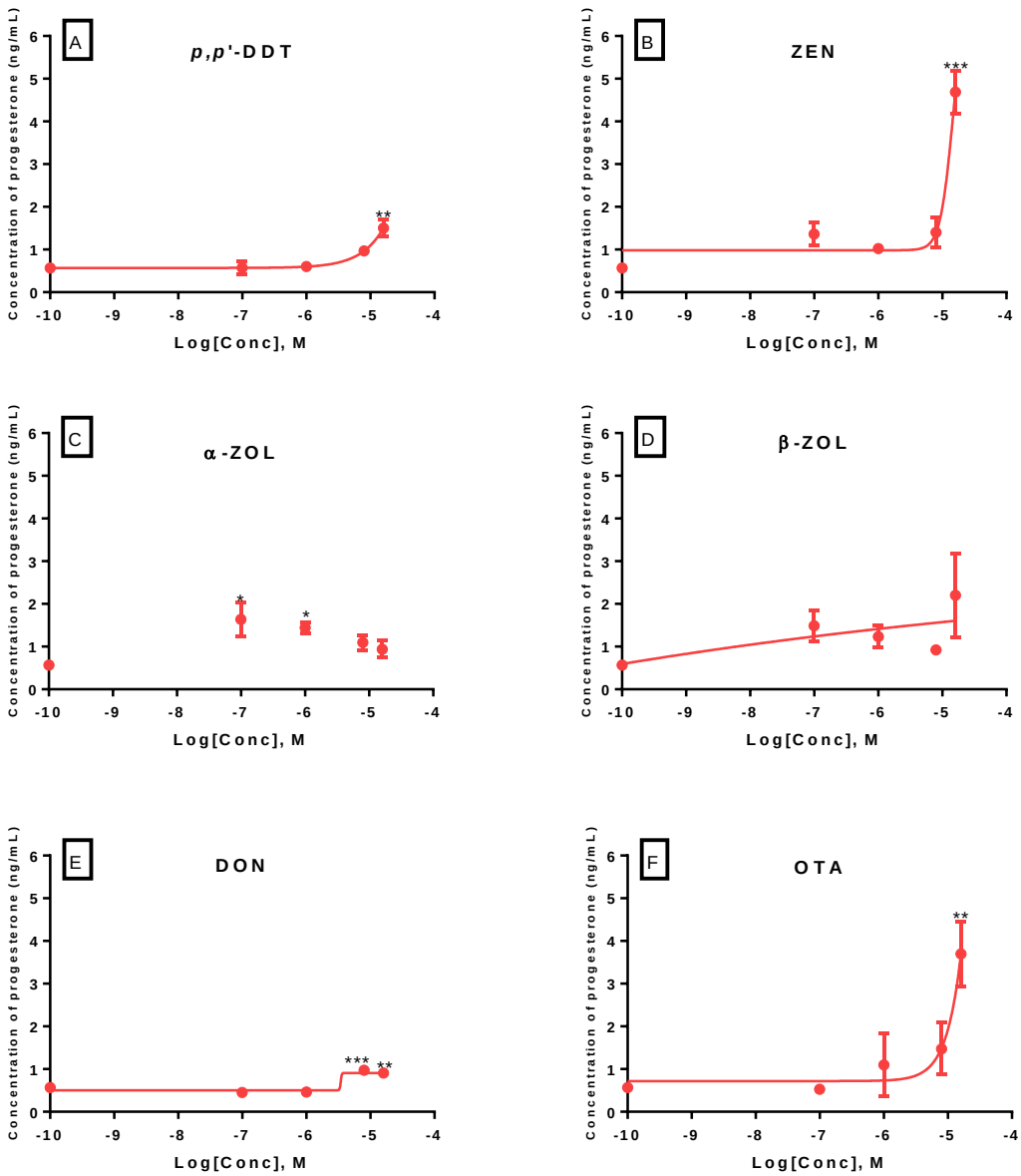

Fig. 4.7: Dose-response curve of the effects of single mycotoxins and pesticides exposure on the progesterone (P4) production in MA-10 Leydig cells. Cells were exposed to the compounds for 48 h and cell media supernatants were collected for P4 analysis using enzyme-linked immunosorbent assay (ELISA). Each value on the graph is the mean of two independent experiments with three technical replicates each and error bars show the standard deviation (SD) of two biological replicates. Data were analysed by comparing treatments and vehicle control using one-way ANOVA and corrected for multiple comparison with Dunnett's test. $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****) represent significant effects. DON: deoxynivalenol; OTA: ochratoxin A; α-ZOL: alpha-zearalenol; β-ZOL: beta-zearalenol; *p,p'*-DDT: 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane

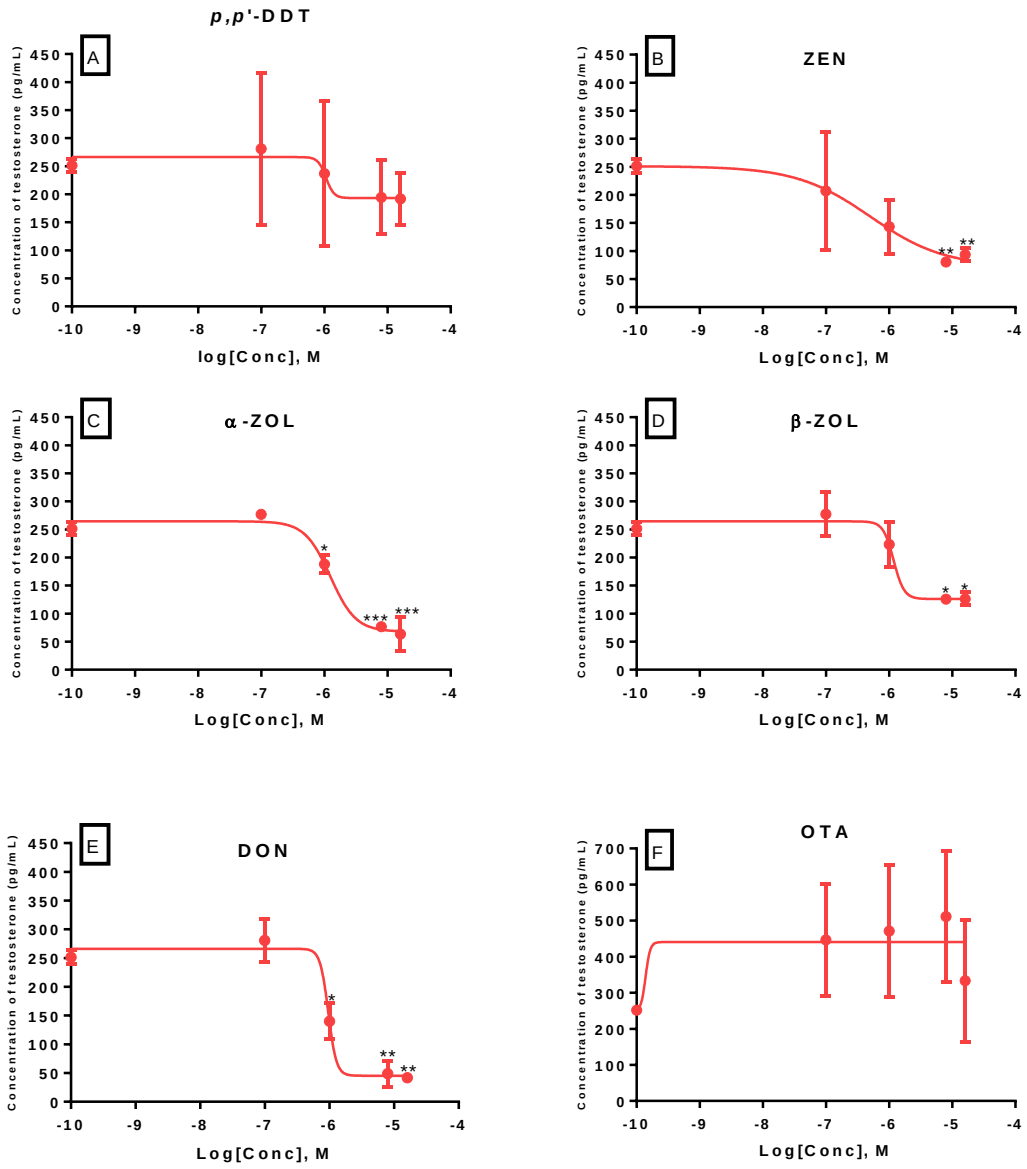

Fig. 4.8: Dose-response curve of the effects of single mycotoxins and pesticides exposure on the testosterone production in MA-10 Leydig cells. Cells were exposed to the compounds for 48 h and cell media supernatants were collected for testosterone analysis using enzyme-linked immunosorbent assay (ELISA). Each value on the graph is the mean of two independent experiments with three technical replicates each and error bars show the standard deviation (SD) of biological replicates. Data were analysed by comparing treatments and vehicle control using one-way ANOVA and corrected for multiple comparison with Dunnett's test. $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****) represent significant effects. DON: deoxynivalenol; OTA: ochratoxin A; α-ZOL: alpha-zearalenol; β-ZOL: beta-zearalenol; *p,p'*-DDT: 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane

4.2.7 Effects of binary mixtures of mycotoxins and pesticide on Leydig cell steroidogenesis

The cytotoxic effects of equimolar binary combinations of mycotoxins and pesticides was first performed, and combinations that showed at least 80% cell viability at 0.1, 1 and 8 μM were chosen to assess the effect of binary combinations of mycotoxins and pesticides on MA-10 Leydig cell steroidogenesis. When *p,p'*-DDT was combined with ZEN, α-ZOL or β-ZOL at equimolar concentrations (0.1 - 8 μM), there was a 1.5 - 3.0 fold increase in P4 secretion, but these effects were not different from the effects observed when cells were treated with *p,p'*-DDT, ZEN, α-ZOL or β-ZOL, alone (**Fig. 4.9A – 4.9C**). Although combination of OTA with ZEN or α-ZOL had stimulatory effect on P4 production at all the doses tested (1.9 - 4.3 fold), these were not different from the effects on P4 release when MA-10 cells were exposed to each of the toxins alone (**Fig. 4.9D and 4.9E**). Combined treatment of ZEN and β-ZOL strongly inhibited P4 release at 0.1 and 1 μM (0.5 fold) and this was significantly reduced compared to the effects each of the toxins exhibited alone (**Fig. 4.9G**). Interestingly, combination of α -ZOL with β-ZOL showed a stimulation of P4 hormone level and this was significantly higher than the effects mediated by each toxin in single treatments (**Fig. 4.9H**).

Testosterone concentration was reduced when *p,p'*-DDT was combined with ZEN, α-ZOL or β-ZOL at equimolar concentrations (0.1 - 8 μM) compared to vehicle control (0.6 - 0.9 fold), and this decline were also significantly different from the level of testostosterone generated after exposure to each of *p,p'*-DDT, ZEN, α-ZOL or β-ZOL, alone (**Fig. 4.10A – 4.10C**). The stimulatory effect of OTA (0.1 - 8 μM) on testostosterone secretion was inhibited by the addition of ZEN or α-ZOL and these effects were additive (**Fig. 4.10D & 4.10E**). In addition, the inhibitory effects observed on testostosterone release when ZEN and α-ZOL were exposed to MA-10 cells at 1 μM alone was not seen in co-treatment of ZEN with α-ZOL indicating antagonistic effects (**Fig. 4F**). On the other hand, the co-treatment of α -ZOL and β-ZOL at 1 μM abolished the significant inhibitory effects on testostosterone production seen when MA-10 cells were exposed to α -ZOL at 1 μM and these interaction was antagonistic at 8 μM (**Fig. 4.10H**).

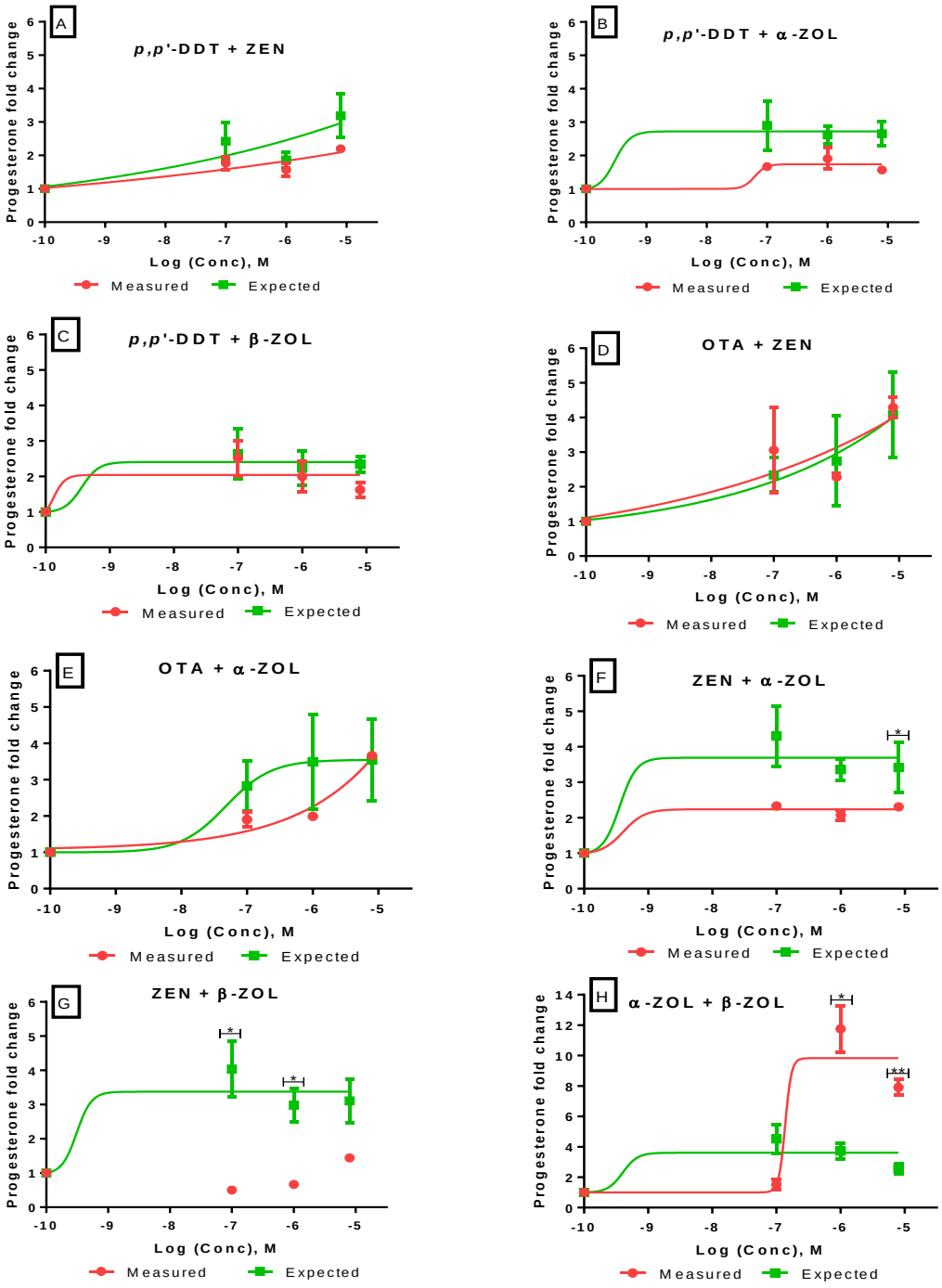

Fig. 4.9: Measured and expected fold change in progesterone (P4) production in MA-10 Leydig cells exposed to binary mixtures of *p,p'*-DDT or OTA with ZEN and its metabolites. Test substances were assayed in triplicate in two independent experiments. Data were analysed with multiple t-test and corrected for multiple comparson using the Holm-Šídák test method. Error bars represent standard deviation of two biological replicates. $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****) represent significant deviation from additive effects.

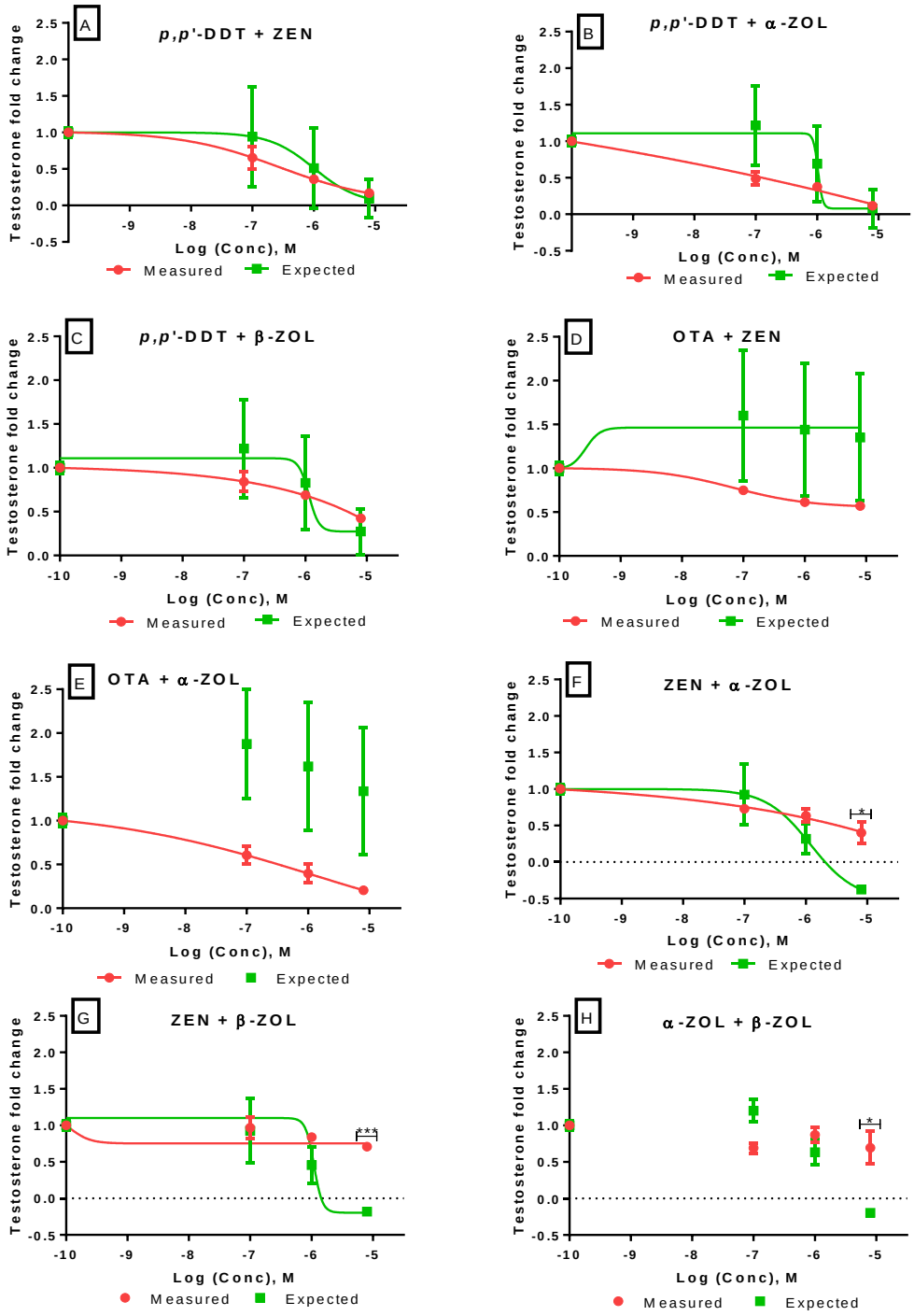

Fig. 4.10: Measured and expected fold change in testosterone production in MA-10 Leydig cells exposed to binary mixtures of *p,p'*-DDT or OTA with ZEN and its metabolites. Test substances were assayed in triplicate in two independent experiments. Data were analysed with multiple t-test and corrected for multiple comparson using the Holm-Šídák test method. Error bars represent standard deviation of two biological replicates. $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****) represent significant deviation from additive effects.

4.3 Discussion

Mycotoxins co-exposure has been reported in food and feedstuff as well as in human exposure studies worldwide. In addition, simultaneous contamination of agricultural products by mycotoxins and pesticides has been reported (Musaiger et al., 2008; Romero-González et al., 2011). Therefore, this study investigated the individual and combined effect of selected mycotoxins (AFB_1, DON, ZEN, α-ZOL, β-ZOL and OTA) and pesticides (*p,p'*-DDT and *p,p'*-DDE) on the viability and steroid hormone production in MA-10 Leydig cells. These mycotoxins and pesticides were selected because they are common contaminants in food, with reproductive toxicity being indicated in previous studies (Ibe et al., 1994; Yang et al., 1997a, b; Uriah et al., 2000; De Jager et al., 2006; Giwercman et al., 2006; Asawasinsopon et al., 2006; Aneck-Hahn et al., 2007; Chakraborty and Verma, 2009; Schoevers et al., 2010; Frizzell et al., 2011; Zatecka et al., 2014).

4.3.1 Cytotoxicity of individual mycotoxins on MA-10 Leydig cells

In the experiments it was found that DON (IC_{50}: 21.97 ± 5.25 µM) was the most cytotoxic mycotoxin tested, followed by OTA (IC_{50} of 28.50 ± 2.63 µM), α-ZOL (IC_{50}: 30.10 ± 0.19 µM) and ZEN (IC_{50}: 60.1 ± 3.59 µM), with limited toxicity for AFB_1 and β-ZOL at the doses tested (**Fig. 4.1**). Cytotoxicity to MA-10 cells has previously been reported for both DON and ZEN, with lower IC_{50} values (0.25 µM and 34 µM) than reported here (Savard et al., 2016). The low IC_{50} reported by Savard et al. is possibly as a result of the very low number of cells used (1 x 10^4 cells/well) and the treatment of the cells in the same day without allowing 24 hours incubation for the cells to recover. DON has been shown to be cytotoxic to other cell types including renal proximal tubule epithelial cells, human lung fibroblast and Chinese hamster ovary K1 cells after 48 hours exposure (Konigs et al., 2007; Ruiz et al., 2011a). Using WST-1 assay, Vejdovszky et al. (2016) showed that DON was highly cytotoxic to Caco2 cells (IC_{50}: 13.0 µM) while ZEN showed lower cytotoxic effect (IC_{50}: 49.5 µM). These IC_{50} values are similar to the ones reported in this study. Lei et al. (2013) reported that DON induced the highest significant cytotoxic effects on PK-15 cell line, followed by AFB_1 and then ZEN. The reduced cell viability caused by DON was speculated to be as a result of inhibition of DNA synthesis (Ranzenigo et al. 2008; Ndossi et al. 2012). However, it is emphasised that the cytotoxicity observed for mycotoxins at

concentrations ≥ 32 μM should be interpreted with caution as human beings are unlikely to be exposed to such concentrations in real life.

Although this assay in the present study was not set up to specifically measure cell proliferation, the results show that ZEN and its metabolites induced MA-10 cell proliferation at 16 μM. This result is similar to that from a panel of reporter gene assay (RGA) cell lines (MMV-Luc, TARM-Luc, TGRM-Luc and TM-Luc) which demonstrated that ZEN induced cell proliferation, but was in contrast to the effect observed in H295R cell line (Frizzell et al., 2011). It was found that for ZEN, α-ZOL and β-ZOL, there was an increase in proliferation at 16 μM dose, but increased toxicity at either 32 μM (α-ZOL) or 64 μM (ZEN and β-ZOL). Savard et al. (2016) also reported that ZEN induced cell proliferation at 10 μM in MA-10 cells. Low concentrations of ZEN and its metabolites (α-ZOL and β-ZOL) are known to induce cell-cycle progression and proliferation in cells that have oestrogen receptors (ER), especially in MCF-7 cells due to their oestrogenic capacity (Dees et al., 1997). However, higher concentrations of ZEN and metabolites were reported to induce apoptosis leading to cell death (Ahamed et al., 2001). It has been reported that ERα/β mRNA and proteins are found in MA-10 Leydig cells (Lin et al., 2014; Milon et al., 2017). Similarly, another Mouse Leydig cell line, BLTK1 (a K1 clone of BLT-1 cells) is also known to express ER-α mRNA (Forgacs et al., 2012). The BLT-1 cells is suggested to have close characteristics with MA-10 cells (Kananen et al., 1996; Rahman and Huhtaniemi, 2004) and therefore, it is possible that the presence of ER in MA-10 cells may have caused the induction of cell proliferation after exposure to oestrogenic ZEN and its metabolites at 16 μM. The significant toxicity of ZEN and its metabolites at higher concentrations could also be attributed to their ability to induce apoptosis through the up-regulation of *Bax* expression, promotion of cytochrome c release in cell cytosol, and activation of caspase-3 and caspase-9 in Leydig cells (Wang et al. 2014c).

OTA cytotoxicity reported here (**Fig 4.1D**) is similar to that for OTA in other cell lines, including Caco-2 cells, mouse RAW264.7 macrophages and Madin-Darby Bovine Kidney (MDBK) cells (Clarke et al., 2014), Vero cells (Bouslimi et al., 2008; Golli-Bennour et al., 2010) and porcine PK15 cells (Klarić et al., 2008, 2012). The cytotoxic effect of OTA, particularly at high concentrations, can be attributed to the fact that OTA

has the ability to inhibit protein synthesis (Creppy et al., 1983), impair mitochondrial phosphorylation (Wei et al., 1985; Aleo et al., 1991); disrupt the nuclear factor-erythroid 2 p45-related factor 2 (Nrf2)-dependent pathway resulting to lipid peroxidation, proteolytic stress and oxidative DNA damage (Cavin et al., 2007, 2009; Limonciel and Jennings, 2014), induce cell apoptosis (Schwerdt et al., 1999; Schilter et al., 2005; Rached et al., 2006; Malir et al., 2016), and dysregulate mitosis (Adler et al., 2009; Mally, 2012). In addition, OTA was recently shown to inhibit the total histone acetyltransferase (HAT) activity of nuclear extracts from the immortalised human kidney epithelial cells (IHKE) in a concentration-dependent manner with an IC_{50} of 24.5 μM (Czakai et al., 2011). However, the actual mechanism through which OTA block HAT activity or the precise group of HATs targeted by OTA have not been established.

In this study, AFB_1 was not cytotoxic to MA-10 cells, with only a minimal increase in toxicity seen above 8 μM. Similar non-cytotoxic behaviour of AFB_1 on Caco-2 and RAW 264.7 cells have been described (Clarke et al., 2014). However, AFB_1 exerted higher toxicity in other studies using intestinal, kidney PK-15 cell line, immune, lung, human umblical vein epithelial, human lung fibroblasts, human bronchial epithelial and Caco-2 cell lines (Liao and Chen, 2005; Mckean et al., 2006a, 2006b; Braicu et al., 2010; Golli-Bennour et al., 2010; Lei et al. 2013; Halbin et al., 2013). The differences in the toxicological effect of AFB_1 in various cells could be attributed to the differences in the capacity of the cell to transform AFB_1 to its toxic metabolite such as aflatoxin-8,9-epoxide. AFB_1 is usually converted to its toxic metabolites through the stimulation of aryl hydrocarbon receptor (AhR) in the liver or extra-hepatic tissues and subsequent activation of cytochrome P450 (CYP450) enzymes, including CYP1A1, CYP1A2, CYP1B1 and CYP3A4 (Mary et al., 2015). Studies indicate that the CYP1A1 and CYP1B1 mRNA and protein are present in MA-10 Leydig cells in relatively low levels (Mandal et al., 2001; Fan et al., 2010; Deb et al., 2011). This is corroborated by the failure of an AhR agonists such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin to induce CYP1B1 mRNA levels in MA-10 cells, but upregulated CYP1B1 mRNA expression in rat liver cells (Deb et al., 2010). Since CYP1A1 and CYP1B1 are regulated by AhR, it is possible that AhR stimulation by AFB_1 does not result in the activation of these CPY450 enzymes in MA-10 cells due to their low levels. Therefore, the low toxicity of AFB_1 observed in MA-10 cells could possibly be due to its non-conversion to toxic metabolites such as AFB1-8,9-epoxide, aflatoxin Q1, aflatoxicol and AFB1-dialdehyde.

4.3.2 Cytotoxic effects of individual pesticides

The oestrogen receptors (ER-α/-β) have been demonstrated in the reproductive system of male rats (Hess et al., 1997a) and men (Akingbemi, 2005). The presence of ER regulate the oestrogen-induced fluid reabsorption during the transfer of spermatids in fluid through the efferent ductules to the epididymis for maturation (Hess et al., 1997b). Inhibition of fluid reabsorption results in the emergence of diluted spermatids which are incapable of normal maturation in the epididymis and therefore, of poor quality (Hess et al., 1997b).

The pesticide *p,p'*-DDT can act through both oestrogen receptor (ER)-dependent and -independent mechanisms (Frigo et al., 2005; Strong et al. 2015). Exposure to *p,p'*-DDT has been associated with the disruption of reproductive organ morphology and function in domestic roosters (Blomqvist et al., 2006). It also has the ability to induce proliferation of ER-competent cells such as breast cancer cells (MCF-7) *in vitro* (Mrema et al., 2012). It should be mentioned that *p,p'*-DDT exposure disrupts ER signalling in the testes; consequently, it has been associated with the disruption of sperm quality (Ayotte et al., 2001; Martin et al., 2002). Its metabolite (*p,p'*-DDE) is a known antiandrogen and androgen receptor antagonist both *in vivo* and *in vitro*, and causes reduction in anogenital distance and increases nipple retention in male rat offspring exposed *in utero* (Kelce et al., 1995). *p,p'*-DDE can also activate the ER and induce cell proliferation in ER-competent cells suggesting it is also a weak oestrogen (Kelce et al., 1995; Strong et al., 2015). This study found that *p,p'*-DDT was cytotoxic to MA-10 cells above 8 µM but limited cytotoxicity was seen with *p,p'*-DDE up to 64 µM.

4.3.3 Cytotoxic and interactive effects of binary mixtures of mycotoxins and pesticides

Few studies have reported the effect of exposure of combinations of mycotoxins to different cell lines and none has reported their cytotoxic and interactive effects on MA-10 Leydig cell line in combination with *p,p'*-DDT and *p,p'*-DDE. In this study, the cytotoxicity of the most common mycotoxins (AFB_1, DON, ZEN, α-ZOL, β-ZOL and OTA) and pesticides (*p,p'*-DDT and *p,p'*-DDE) on MA-10 Leydig cells were evaluated in their binary mixtures.

Although no longer used in most developed countries due to its known toxicity, *p,p'*-DDT is used officially for disease vector control in some tropical countries, and may be misused in agriculture in certain places. This raises the possibility of co-exposure of humans to *p,p'*-DDT and mycotoxins. In our experiments, the combination of *p,p'*-DDT with mycotoxins mostly increased the cytotoxicity of the mycotoxin compared to the mycotoxin alone. In contrast, the combination with *p,p'*-DDE has generally not increased mycotoxin cytotoxicity. In the presence of DON or OTA, the proliferative effects of ZEN, α-ZOL and β-ZOL were no longer evident, but AFB_1 did not prevent the proliferation induced by certain doses of ZEN or its metabolites. Interestingly, DON cytotoxicity was reduced in the presence of equivalent doses of AFB_1, showing that the results of interactions between mycotoxins can be complex.

AFB_1 + OTA showed induction in cell proliferation at 0.1 - 8 µM but was cytotoxic at 16 - 64 µM combinations, although this was not different to the cytotoxicity of OTA alone at these doses. Clarke et al. (2014) reported significant cytotoxic effect when MDBK cells were exposed to binary mixtures of AFB_1 and OTA after 48 hours of exposure but no cytotoxicity for RAW 264.7 and Caco-2 cells exposed to the same concentration. Additive cytotoxic effect was observed after exposure of AFB_1 + OTA to Green Monkey Vero cells (Golli-Bennour et al., 2010) and HepG2 cells (Corcuera et al., 2011). The results of this present study (**Fig. 4.4B**) has shown that the combination of AFB_1 and DON gave lower cytotoxicity than DON alone. The interactive effects suggests that antagonistic effetcs could have resulted in MA-10 cells exposed to AFB_1 to be less susceptible to DON induced cytotoxicity than cells not exposed to AFB_1. However, it is difficult to speculate on the possible mechanism for such antagonistic interaction. Nevertheless, similar findings were seen for AFB_1 and ZEN or its metabolites, particularly at 0.1 µM – 16 µM. This contrasts with an additive effect of AFB_1 and DON on *Cyprinus carpio* primary hepatocytes (He et al., 2010) and a synergistic effect on PK-15 cells (Lei et al. 2013) that have been previously reported. The later study also reported synergy for AFB_1 + ZEN, which was not seen in the MA 10 cells in our study (Lei et al., 2013).

4.3.4 MA-10 Leydig cell line produces progesterone and testosterone in both unstimulated and stimulated condition

Most studies investigating the effects of chemicals interfering with steroidogenesis have applied the H295R cell line for the assessment of adrenal production of 17β-oestradiol (E2) and testosterone (Hecker et al., 2006, 2011; Pinto et al., 2018). Arguably, the H295R steroidgenesis assay identify impairments in adrenal steroidogenesis, but fails to accurately determine perturbations in testicular steroidogenesis (Strajhar et al., 2017). Therefore, we applied MA-10 Leydig cells to examine the effects of xenoestrogens on testicular P4 and testosterone production. In this study, we have shown that the MA-10 Leydig cell line can produce substantial amounts of P4 and testosterone both in basal and stimulated condition using our cell culture and treatment modifications. MA-10 cells produced basal P4 and testosterone levels (0.60 ± 0.07 ng/mL and 251.6 ± 20.7 pg/mL, respectively) whereas 8-Br-cAMP-stimulated P4 and testosterone levels were 135.50 ± 44.34 ng/mL and 5233.9 ± 594.63 pg/mL, respectively. This is similar to previous studies which reported that 8-Br-cAMP (100 μM) treated MA-10 Leydig cells had increased testosterone concentration in the medium up to 3200 ± 40 pg/ml, and expressed mRNA for steroidogenic enzymes, including StAR, Cyp17a1, Cyp11a1, 3β-hsd1 and 17β-hsd3 (Dankers et al., 2013; Roelofs et al., 2014, 2015). In a different study, induction of BLTK1 Leydig cell line with 10 μM of forskolin indicated that maximum P4 level (200 ng/mL) occurred at 4 h after exposure whereas the highest level of testosterone (230 pg/mL) was detected after 48 h of exposure (Forgacs et al., 2012). With regard to the basal and stimulated concentrations of testosterone in MA-10 Leydig cells in our study, MA-10 cell line is more suitable for the assessment of the effect of toxicants on testicular testosterone production.

4.3.5 OTA modulates progesterone and testosterone production in MA-10 Leydig cells

The result of this study indicate that OTA is a potential endocrine disruptor as it interferes with testicular steroidogenesis. The present study (Fig. 4.7F) shows that OTA significantly increased P4 level in MA-10 cells in dose-dependent manner after 48 h exposure. This finding is in accordance with a previous study in which 100 ng/mL of OTA significantly increased P4 production and upregulated 3β-hydroxysteroid

dehydrogenase type 1 (3β-HSD1) mRNA and protein levels in JEG-3 placental cell line (Woo et al., 2013). However, the finding is in contradiction with previous study using H295R cell line where exposure to OTA at 0.1 ng/mL to 1000 ng/mL did not alter P4 production, but significantly induced oestradiol secretion (Frizzell et al., 2013). The discrepancy seen in the P4 secretion of H295R cells exposed to OTA could be as a result of 3β-hydroxysteroid dehydrogenase (3β-HSD) activity. The 3β-HSD enzyme is mainly involved in the conversion of 3β-hydroxy-5-ene-steroids (dehydroepiandrosterone, pregnenolone) to 3-oxo-4-ene-steroids (androstenedione, P4) in the gonads, placenta and adrenal cells (Woo et al., 2013). The 3β-hydroxysteroid dehydrogenase type 1 (3β-HSD1) is commonly found in JEG-3 (Woo et al., 2013) and MA-10 cells (Roelofs et al., 2014, 2015; Dankers et al., 2013) whereas 3β-hydroxysteroid dehydrogenase type 2 (3β-HSD2) is the major 3β-HSD type expressed in H295R (Hilscherova et al., 2004; Ndossi et al., 2012).

The present study also shows that OTA (0.1 - 8 μM) significantly increased testosterone level (˜2-fold) in MA-10 cells after 48 h exposure (Fig. 4.8F). The result of our study is similar to the work of Gharbi et al. (1993) who reported that exposure of male rats to OTA (2 mg/kg per day) for 8 weeks resulted in a 2-fold increase of testicular testosterone level. Similarly, feeding of male rats with OTA-contaminated feed (1 mg/kg) for 6 months caused induction in serum follicle stimulating hormone (FSH), testosterone, triiodothyronine 3 (T_3) and thyroxine (T_4), and a reduction of LH levels (Hassan et al., 2010). In contrast to our study, a decline in both serum cortisol and testosterone levels were observed in male rats exposed to OTA (4 mg/kg) in feed for 30 days (Kumar et al., 2011), and there was a reduction in testosterone levels in dispersed interstitial cells obtained from the testis of adult gerbils after exposure to 1838 μM of OTA (Fenske and Fink-Gremmels, 1990), which is over 100-fold more than the highest concentration of OTA applied in our study. It can be deduced that reduction in testosterone levels was only observed in animal and *in vitro* studies applying very high concentration of OTA, and therefore the human relevance of such studies are questionable. In a recent study, OTA at 1000 ng/mL had no significant effect on testosterone production in H295R cell line compared to vehicle control after 48 h of exposure (Frizzell et al., 2013). The variations in the effect of OTA in the testosterone secretion in MA-10 cells and H295R cells could possibly be as a result of difference in

cell origin, cell number, and the media composition. In addition, it is possible that OTA is metabolised in MA-10 Leydig cells to a potent metabolite which is capable of inducing P4 and testosterone, and therefore, this needs to be investigated in future studies. Other studies demonstrated that OTA was present in the seminal fluid of boars exposed to OTA and caused decline in sperm production and poor sperm quality, but did not affect Leydig cell morphology (Solti et al., 1999; Biró et al., 2003). The decrease in sperm production and abnormal sperm quality observed in male animals exposed to OTA can be explained by its ability to interfere with protein and DNA synthesis leading to the production of sperm cells with unstable membranes (Solti et al., 1999; Biró et al., 2003). Considering that serum OTA levels at 1,800 ng/mL (4.458 µM) and 1,136 ng/mL (2.813 µM) (Steyn, 1993; Maaroufi et al., 1995) have been detected in human exposure studies, these findings may have clinical relevance as exposure in the early developmental period might lead to testicular dysgenesis syndrome in exposed foetus.

4.3.6 Zearalenone and metabolites α-zearalenol and β-zearalenol differentially target progesterone and testosterone secretion in MA-10 Leydig cell line

ZEN and its metabolites are known as endocrine disruptors at the level of ER and PR signalling, and hormone production (Frizzell et al., 2011). In this study (**Fig. 4.7B 4.7D**), we have shown that ZEN and its metabolites treatment for 48 h increased the ability of MA-10 cells to produce P4 in cell media supernatants ($p \leq 0.01$). Similar concentrations of ZEN, α-ZOL and β-ZOL (0.1 - 10 µM) employed in our study also significantly induced P4 levels in unstimulated H295R cell line after 48 h exposure (Frizzell et al., 2011). However, ZEN (10 µM) had no significant impact on basal P4 levels in MA-10 cells, but significantly decreased forskolin-stimulated P4 production (Savard et al., 2016). It must be noted that Savard et al. (2016) used smaller number of cells (2.5×10^4) and exposed cells to ZEN for only 6 h, and these may have caused the discrepancies observed in P4 levels. In the presence of IGF1 and FSH, α-ZOL had no effect on P4 secretion, but decreased P4 level when co-exposed with only FSH in bovine large follicular granulosa cells (Pizzo et al., 2015). These results are in agreement with another *in vitro* studies (Tiemann et al., 2003), which showed that ZEN, α-ZOL and β-ZOL down regulated *CYP11A1* and *3β-HSD* mRNA expression in porcine granulosa cells and therefore, inhibited P4 secretion induced by FSH plus IGF1. In

contrast, α-ZOL significantly increased FSH plus IGF1-induced P4 production in porcine granulosa cells (Ranzenigo et al., 2008; Cortinovis et al., 2014). In unstimulated small bovine granulosa cells, β-ZOL stimulated both P4 and E2 production whereas α-ZOL had no effect on these hormones in the presence of FSH alone, indicating that α-ZOL and β-ZOL have biphasic effects (Pizzo et al., 2016). This variation in P4 production could be as a result of the application of a stimulated and an unstimulated cell model, and/or the differences in the cell type or oestrogenic potency. For instance, E2 had stimulatory effect on P4 secretion in porcine granulosa cells (Veldhuis et al., 1985) whereas it caused decline in P4 production in bovine granulosa cells (Langhout et al., 1991). It has also been shown that increased P4 production in conjunction with decreased E2 level is common in follicular atresia, especially in pigs (Guthrie et al., 1993; Pan et al., 2012). Therefore, an increased P4 level combined with low testosterone level could result in male germ cell degeneration.

In males, testosterone level is critical in spermatogenesis, sperm maturation, and sexual function in adults and essential for the masculinisation of the male foetus *in utero* (Akingbemi et al., 2005). The dysregulation of testosterone biosynthesis in both foetal and adult Leydig cells can cause subsequent sub-fertility or infertility (Skakkebaek et al., 2016). For instance, male neonatal mice whose mothers were exposed to ZEN (1.3, 3.9 or 6.6 mg/kg/day) or mono-(2-ethylhexyl)-phthalate (9.2; 46.3 or 92.7 mg/kg/d) during the pre-conception period had abnormal testicular gene signatures which were suggestive of maternal developmental reprogramming and this could lead to the disruption of key genes involved in testicular development and spermatogenesis in adulthood (López-Casas et al., 2012). In this study (**Fig. 4.8A 4.8D**), it was found that ZEN, α-ZOL and β-ZOL significantly inhibited testosterone production ($p \leq 0.01$) in MA-10 Leydig cells in dose-dependent fashion compared to vehicle control. The decreased production of testosterone, and the increased induction of P4 secretion in the MA-10 Leydig cell line exposed to ZEN and its metabolites could be either as a result of inhibition of Cyp17a1 enzyme involved in the conversion of P4 to testosterone or the upregulation of the 5α-reductase type 1 (srd5α1) enzyme involved in converting testosterone to DHT. In addition, the reduction of testosterone level in MA-10 cells exposed to ZEN and α-ZOL could be due to increased activity or upregulation in mRNA level of *CYP19A1* (aromatase) which is responsible for the conversion of testosterone to E_2. Previous studies show that ZEN and its metabolites inhibited

testicular testosterone secretion, inhibited spermatogenesis, decreased sperm quality, impaired sperm DNA integrity, induced germ cell degeneration, infertility and caused perturbation of the genes for enzymes involved in steroidogenesis and ATP-binding cassette efflux (ABC) transporters *in vivo* and *in vitro* (Berger et al., 1981; Kim et al., 2003; Yang et al., 2007a, 2007b; Benzoni et al., 2008; Tsakmakidis et al., 2008; Salah-Abbès et al., 2009; Minervini et al., 2010; Boeira et al., 2012, 2014; Koraichi et al., 2013; Li et al., 2014b; Liu et al., 2014; Wang et al., 2014d; Cheraghi et al., 2015; Lin et al., 2015; Zheng et al., 2016; Adibnia et al., 2016; Bielas et al., 2017; Pang et al., 2017; Yang et al., 2017). In contradiction to the finding of our studies, Frizzell et al. (2011) reported that ZEN, α-ZOL and β-ZOL significantly increased testosterone levels in H295R cell line after 48 h, particularly at 10 μM. In another *in vitro* study using H295R cell line, 48 h exposure to ZEN significantly induced testosterone secretion with maximum induction at 10 μM (Kolle et al., 2012). It is speculated that the H295R steroidogenesis assay determines alterations in adrenal steroidogenesis, and may not accurately determine perturbations in testicular steroidogenesis (Strajhar et al., 2017). This is due to low levels of testosterone produced by H295R cells even though it contains all the adrenal steroidogenic enzymes and also, the Nu-serum in H295R cells growth media contribute to substantial concentration of P4 and testosterone which may give false increase in P4 and testosterone levels in the assay (Zhang et al., 2011; Nakano et al., 2016; Strajhar et al., 2017). Principato et al. (2018) demonstrated that 17 out of 20 chemicals (85%) which had no effect on testosterone secretion in the H295R steroidogenesis assay were found to inhibit testosterone production in purified Leydig cells (Principato et al., 2018). In subsequent study, Zhang et al. (2011) suggested that testosterone should not be used as a reliable marker of toxicity in the H295R assay as a result of the presence of 1260 pg/mL of T in the untreated H295R culture media which gives a false positive increase in testosterone production. Similarly, 1880 pg/mL of testosterone was detected in the untreated H295R culture media containing 2.5% Nu-serum whereas 161.6 pg/mL of testosterone was detected in H295R cells grown in the absence of Nu-serum after 48 h, confirming testosterone contamination of media through the addition of Nu-serum (Strajhar et al., 2017). In our study, we diluted mycotoxins and pesticides with DMEM/F12 media containing only 5% charcoal-stripped foetal bovine serum to ensure that treatment media are free from exogenous hormone. Our result highlights the importance of using reproductive cell lines in the

assessment of the effects of xenoestrogens on steroidogenesis to complement H295R steroidogenesis assay.

4.3.7 Effects of DON on Leydig cell steroidogenesis

The present study (**Fig. 4.6 & Fig. 4.7**) shows the differential effects of DON on Leydig cell steroidogenesis. DON inhibited P4 concentration at low concentrations (0.1 and 1 μM) and significantly increasedd P4 secretion ($p \leq 0.01$) at 8 and 16 μM after 48 h exposure, even though MA-10 cell viability were below 80% at 8 and 16 μM. Similar doses of DON (3.4 μM) applied in our study also stimulated P4 release in porcine granulosa cells (Medvedova et al., 2011; Kolesarova et al., 2012) and H295R cells (Ndossi et al., 2012). In a more recent study, *in vitro* exposure to DON (4 - 16 μM) also modulated P4 and E2 production in porcine ovarian granulosa cells (Kolesarova et al., 2017). However, DON (4 - 16 μM) in the presence of FSH reduced P4 release in the same porcine ovarian granulosa cells (Kolesarova et al., 2017). It is well established that DON inhibits P4 secretion in cells stimulated with FSH or IGF1. For instance, co-exposure of DON with either FSH or IGF1 or both inhibited P4 release in porcine granulosa cells *in vitro* (Ranzenigo et al., 2008; Cortinovis et al., 2014) and MA-10 Leydig cells (Savard et al., 2016). This present study (Fig. 4.7E) also report that DON (1 - 16 μM) strongly inhibited testosterone production in MA-10 cells ($p \leq 0.01$). In agrement to our study, DON at 3.4 μM also inhibited testosterone levels in H295R cell line (Ndossi et al., 2012). Male rats exposed to DON (5 mg/kg/day) for 28 days had decline in serum testosterone levels, testicular germ cell degeneration, decrease in absolute cauda epididymal sperm numbers, and reduction in caudal epididymal weights (Sprando et al., 2005). In human epidemiological studies, levels of urinary DON and its conjugates (1.471 - 4.178 μM) have been detected in pregnant women and therefore, the concentrations causing hormonal dysreulation have clinical relevance.

4.3.8 Effects of *p,p′*-DDT on Leydig cell steroidogenesis

The present study (**Fig. 4.7A**) have shown that exposure of MA-10 cells treated with 8 μM and 16 μM of *p,p′*-DDT for 48 h significantly ($p \leq 0.001$) increased P4 synthesis. It is well established that *p,p′*-DDT and its metabolites act as endocrine disruptors through the alteration of steroidogenic pathway, receptor mediated changes in protein synthesis, and anti-androgenic and oestrogenic activity (Kelce et al., 1995; Crellin et al., 1999;

Wójtowicz et al., 2007a). Studies using different cells have demonstrated that *p,p'*-DDT alters P4 synthesis. For instance, *p,p'*-DDT stimulated basal P4 production in rat granulosa-luteal cells after 48 h exposure at doses similar to the ones that increased basal MA-10 Leydig cell P4 release in our study (Nejaty et al., 2001). In agreement with the findings of our study, DDT isomers (*p,p'*-DDT, *p,p'*-DDE, *o,p'*-DDT and *o,p'*-DDE) at 1 - 1000 ng/mL significantly increased P4 hormone levels in JEG-3 human choriocarcinoma cells after 72 h of treatment (Wójtowicz et al., 2007a). In subsequent studies, *p,p'*-DDT (100 and 1000 ng/mL) did not affect P4 secretion whereas *p,p'*-DDE, *o,p'*-DDT and *o,p'*-DDE at the same concentrations stimulated P4 secretion (Wójtowicz et al., 2007b). However, this was only a short term exposure of 24 hours, indicating that *p,p'*-DDT induction of P4 hormone level occurs beyond 24 h exposure. The modulation of P4 secretion by *p,p'*-DDT and its metabolites can be as a result of alteration of cAMP synthesis, *CYP11A* mRNA expression and CYP19A1 activity in the exposed cells (Crellin et al., 1999; Wójtowicz et al., 2007b; Sanderson et al., 2002).

In this study (**Fig. 4.8A**), the presence of 8 µM and 16 µM of *p,p'*-DDT decreased testosterone synthesis, although these reductions were not significantly different ($p > 0.05$) from the level found in the vehicle control at 48 h of exposure. It has been reported that exposure of male rats to the *p,p'*-DDT metabolite *p,p'*-DDE (200 mg/kg per day) by gavage for 4 days significantly reduced both seminal vesicle and prostate weight, and blocked androgen receptor response (Kelce et al., 1995). However, the serum testosterone concentration in the exposed rats remained unchanged (Kelce et al., 1995). In a different study, mixtures of endocrine disruptors containing *p,p'*-DDE caused significant abnormalities in the testis and epididymis, and decreased epididymal sperm count in exposed male rats whereas no significant effects were found in either testosterone or inhibin B hormone levels in the rats (Isling et al., 2014). In subsequent studies, rats exposed to *p,p'*-DDT metabolite *p,p'*-DDE at 100, 200, and 300 mg/kg/day, had reduced DHT and increased E2 levels, but serum testosterone, prolactin, LH, and FSH remained unaffected (O'connor et al., 2002), further confirming our findings. The results of our study (**Fig. 8A**) were also in accordance to several human epidemiological studies which reported no reduction and/or no changes in testosterone, FSH or LH hormone levels in Swedish and Latvian men (Hagmar et al., 2001), in previous *p,p'*-DDT spray-workers (Cocco et al., 2004) and Swedish fishermen (Rignell-Hydbom et al., 2004). However, our findings do not concur with other human epidemiological

studies reporting that exposure to *p,p'*-DDT and its metabolites was significantly associated with lower testosterone concentrations (Martin Jr et al., 2002; Haugen et al., 2011; Blanco-Muñoz et al., 2012). The variations in the associations of *p,p'*-DDT exposure with testosterone levels could possibly be as a result of differences in exposure concentrations, duration, pathways/routes and study populations/species. It must be noted that *p,p'*-DDT concentrations relevant to the levels used in this study (40.31 μM) have been reported in human serum samples from India (Dua et al., 2001).

4.3.9 Effects of binary combinations of mycotoxins and pesticide *p,p'*-DDT on Leydig cell steroidogenesis

Despite the fact that humans and animals alike can be exposed to a range of mixtures of environmental and food contaminants (including mycotoxins and pesticides), most toxicological studies have only taken into account the effects of exposure to a single contaminant. In addition, they are often 'boxed' to their respective contaminant group (mycotoxins or pesticides), with limited studies investigating mixtures of chemical contaminants co-occurring together spanning different chemical groups. Very limited data is available on the combined toxic effects or the 'cocktail effect' of exposure to mycotoxins and/or persistent organochloride pesticides, thus the ability to accurately assess the risks to health of combined exposure is currently inadequate. This is the first study reporting the effects of mycotoxins and pesticide combinations on Leydig cell hormone production. This study has shown that co-exposure of *p,p'*-DDT with ZEN, α-ZOL or β-ZOL caused alteration on Leydig cells steroidogenesis, although effects were similar to those observed after exposure to the each of the toxins alone (**Fig 4.9A - 4.9C & Fig. 4.10A - 4.10C**). Co-exposure of α-ZOL and β-ZOL strongly modulated P4 secretion which was significantly different ($p \leq 0.01$) compared with either α-ZOL or β-ZOL, alone (**Fig. 4.9H**). This fact could lead to the conclusion that the effects observed after co-exposure to α-ZOL and β-ZOL are due to the interaction occurring between the two mycotoxins. This is possibly due to the ability of both α-ZOL and β-ZOL to activate ER transcriptional activity and increase PR mRNA and protein expression (Frizzell et al., 2011) as the MA-10 cell line expresses both E2 and P4 receptor (Milon et al., 2017). In bovine small-follicle granulosa cells, co-treatment of α-ZOL and β-ZOL to similar concentrations used in our study had inhibitory effect on E2 production, but had no significant effect on P4 production. This variation could be as a result of the

stimulation of bovine granulosa cells with IGF1 prior to α-ZOL and β-ZOL co-exposure. These findings also show that combination of *p,p'*-DDT with ZEN, α-ZOL or β-ZOL significantly inhibited Leydig cell testostosterone production when combined at equimolar concentrations (0.1 - 8 μM) compared to vehicle control, and these decline was also significantly different from the level of testostosterone generated after exposure to each of *p,p'*-DDT, ZEN, α-ZOL or β-ZOL, alone. It can be speculated that co-treatment of ZEN, α-ZOL or β-ZOL with *p,p'*-DDT enhances their toxic effects on testostosterone synthesis in Leydig cells which poses a public health threat to male reproductive health.

4.4 Conclusion

The results of this study indicate that exposure to DON and OTA in particular are cytotoxic to MA-10 Leydig cells, and this cytotoxicity is enhanced by co-exposure with either *p,p'*-DDT or *p,p'*-DDE. In addition, the results presented here show that MA-10 Leydig cell line is a useful model for complementing H295R cell line in assessing the effects of xenoestrogens on testicular steroidogenesis. The findings of this study also indicate that exposure to *p,p'*-DDT, ZEN, α-ZOL, β-ZOL, OTA or DON at concentrations relevant to human exposure could cause dysregulation of Leydig cell steroidogenesis, especially during the critical developmental periods. Furthermore, the effects of ZEN, α-ZOL and β-ZOL on MA-10 Leydig cell steroidogenic function is enhanced by co-exposure with *p,p'*-DDT pesticide, further compounding the threat posed by these mycotoxins to male reproductive health. Theses raise the possibility that such exposures could contribute to adverse male reproductive health in exposed populations. However, human epidemiological studies is required to establish a causal relationship between mycotoxins and/or pesticide exposure on human reproductive and development health.

Chapter 5

The *in vitro* effects of individual and chemically-defined mixtures of regulated mycotoxins and persistent organochloride pesticides on the viability, hormone production and gene expression in BeWo human placental cell line

5.1 Introduction

Mycotoxins are common contaminants of agricultural produce, especially in low and middle income countries, and it has been estimated that 25% of the world's food crops are contaminated by mycotoxins (IARC, 2002). In addition, contamination levels of *p,p'*-DDT pesticide are usually higher in regions where *p,p'*-DDT is still used for malaria control (Bouwman et al., 2011). Human exposure to mycotoxins and *p,p'*-DDT pesticide could result in reproductive and developmental toxicity as these compounds are transported across the placenta by transport proteins and these toxins can be found in placental tissues, amniotic fluid and/or cord blood (Cortinovis et al., 2013; Murray et al., 2018). Exposure to these compounds *in utero* can result in adverse pregnancy and birth outcomes since the embryo or foetus is vulnerable to assault, especially at the critical windows of development. The major placental hormones, E2, P4, and β-hCG are important in the regulation that takes place in the maternal and foetal interface during pregnancy (Cross, 2006). In early pregnancy, β-hCG is produced by the placental trophoblasts to ensure proper embryo implantation and it is a major biomarker of normal trophoblast function (Prouillac and Lecoeur, 2010). Increased levels of this hormone have been associated with pre-eclampsia. Similarly, oestrogens play major roles in the formation of syncytiotrophoblasts, cellular differentiation, trophoblast invasion, nutrient exchange as well as uteroplacental blood flow during pregnancy. A dysregulation of oestrogen biosynthesis may negatively impact on the development of the foetus and placenta, and result in altered foetal development, low birth weight, small head circumference, pre-eclampsia, eclampsia, preterm birth, stillbirth, and intrauterine growth restriction (Cartwright et al., 2010). On the other hand, P4 is mainly involved in the proliferation, secretory functions, regression and regeneration of the endometrium as well as facilitating the correct signal required for embryo implantation and healthy pregnancy (Prouillac and Lecoeur, 2010). Therefore, disruption of P4 levels is

associated with poor embryo implantation, and subsequent preterm labour and spontaneous abortion (Guibourdenche et al., 2009). Placental perturbation in early pregnancy is also associated with long term pathological effects in adulthood, including cardiovascular diseases, obesity, osteoporosis and type 2 diabetes (Ravelli et al., 1998; Barker et al., 2004; De Boo et al., 2006; Prouillac and Lecoeur, 2010; Langley-Evans and McMullen, 2010; Roseboom et al., 2011; Guttmacher et al., 2014; Wang et al., 2017). Chronic exposure to mycotoxins and persistent organochloride pesticides occur from *in utero* through to childhood and into adulthood, and could likely be the cause of growth impairment and poor reproductive health outcomes as well as susceptibility to adult diseases common in areas with high exposures. Although mycotoxins and persistent organochloride pesticides can be transferred across the placenta and could affect placental endocrine function and foetal development, *in utero* exposure and foetal risk assessment are still inadequate. In addition, studies assessing the potential combination effects of mycotoxins and persistent organochloride pesticides on reproduction are very scarce.

Mycotoxins and *p,p'*-DDT pesticide exposure may also affect reproduction and development through epigenetic reprogramming and genomic imprinting. DNA methylation and genomic imprinting play critical roles in growth, morphology and nutrient transfer capacity of the placenta, therefore, are important in normal embryonic implantation, foetal and placental growth, and development (Robertson and Wolffe, 2000). The perturbation of placental DNA methylation and imprinted genes in humans by environmental exposures has been linked to developmental abnormalities such as intrauterine growth restriction, low birth weight, spontaneous abortion or foetal death *in utero* as well as overweight, obesity, type 2 diabetes and cardiovascular diseases in adulthood (Moore et al., 2015). Developmental programming in the critical windows of *in utero* development relies heavily on epigenetic mechanisms including DNA methylation and histone modifications, and can potentially have negative consequences for subsequent developmental disorders and disease manifesting either in childhood or adulthood (Gluckman *et al.* 2008). This is true as changes in methylation pattern in the placenta and foetus has been linked to adverse placental morphology and poor birth outcomes (Banister et al., 2011). For instance, differential DNA methylation of fibroblast growth factor-12 (*FGF12*) and insulin growth factor 1 receptor (*IGF1*R) genes were observed in the white blood cells from Gambian infants whose mothers

were exposed to AFs during early pregnancy (Hernandez-Vargas et al., 2015), indicating that exposure to environmental and food contaminants could have negative epigenetic effects on humans. However, the molecular mechanisms that underlie these observations remain unclear and requires further elucidation.

Different placental cell lines have been used for the study of toxicological effects of xenobiotics on the placenta, including BeWo, JEG-3 and JAr cell lines (Prouillac and Lecoeur, 2010). However, the BeWo placental cell line was chosen for this study as it has been recommended as a useful model for assessing the impacts of environmental and food toxins on the morphological and biochemical characteristics of the placenta (Prouillac et al, 2009, 2012). The BeWo cell line is known to non-spontaneously differentiate into syncytiotrophoblast, and exhibit the morphology and functions similar to the normal human trophoblast, including the production of endocrine hormones (Utoguchi et al., 2000; Prouillac et al, 2009; 2010). Firstly, this study evaluated the cytotoxic effects of mycotoxins (AFB_1, DON, ZEN, α-ZOL, β-ZOL and OTA) and persistent organochloride pesticides (*p,p'*-DDT and *p,p'*-DDE), either in single or binary mixtures, on BeWo cells. In addition, the effect of single and/or binary mixtures of mycotoxins and *p,p'*-DDT pesticide on placental endocrine function was examined. The effect of ZEN and *p,p'*-DDT, either in single or in mixtures, on placental gene expression were further explored using RT-qPCR.

5.2 Results

5.2.1 Effects of individual mycotoxins and pesticides on cell viability

The results of the cell viability experiment of BeWo cells exposed to single mycotoxins and pesticides at 0.1 µM to 64 µM for 48 h determined with MTT assay are illustrated in **Fig. 5.1**. Dose-dependent reduction of cell viability was observed, especially in DON and OTA exposure compared to 0.1% DMSO control (**Fig. 5.1C & 5.1D**). According to the IC_{50} values obtained for single mycotoxin treatment, OTA (IC_{50}: 10.21 ± 0.21 µM) showed the highest cytotoxic effect on BeWo cells followed by DON (IC_{50}: 13.39 ± 1.12 µM), β-ZOL (23.71 ± 0.05 µM), and ZEN (IC_{50}: 33.97 ± 2.25 µM). At doses below 16 µM, ZEN and β-ZOL had no cytotoxic effect on BeWo cells but significantly reduced cell viability ($P \leq 0.001$) at 32 µM and 64 µM with the highest reduction in cell viability occurring at 64 µM (25.1% and 44.4%, respectively; **Fig. 5.1E and 5.1H**). The IC_{50} values of AFB_1 and α-ZOL were above the highest concentration tested (64 µM)

and therefore, could not be determined. Among the pesticides applied, *p,p'*-DDT was more cytotoxic to BeWo cells than *p,p'*-DDE, with IC_{50} values of 26.60 ± 1.97 µM.

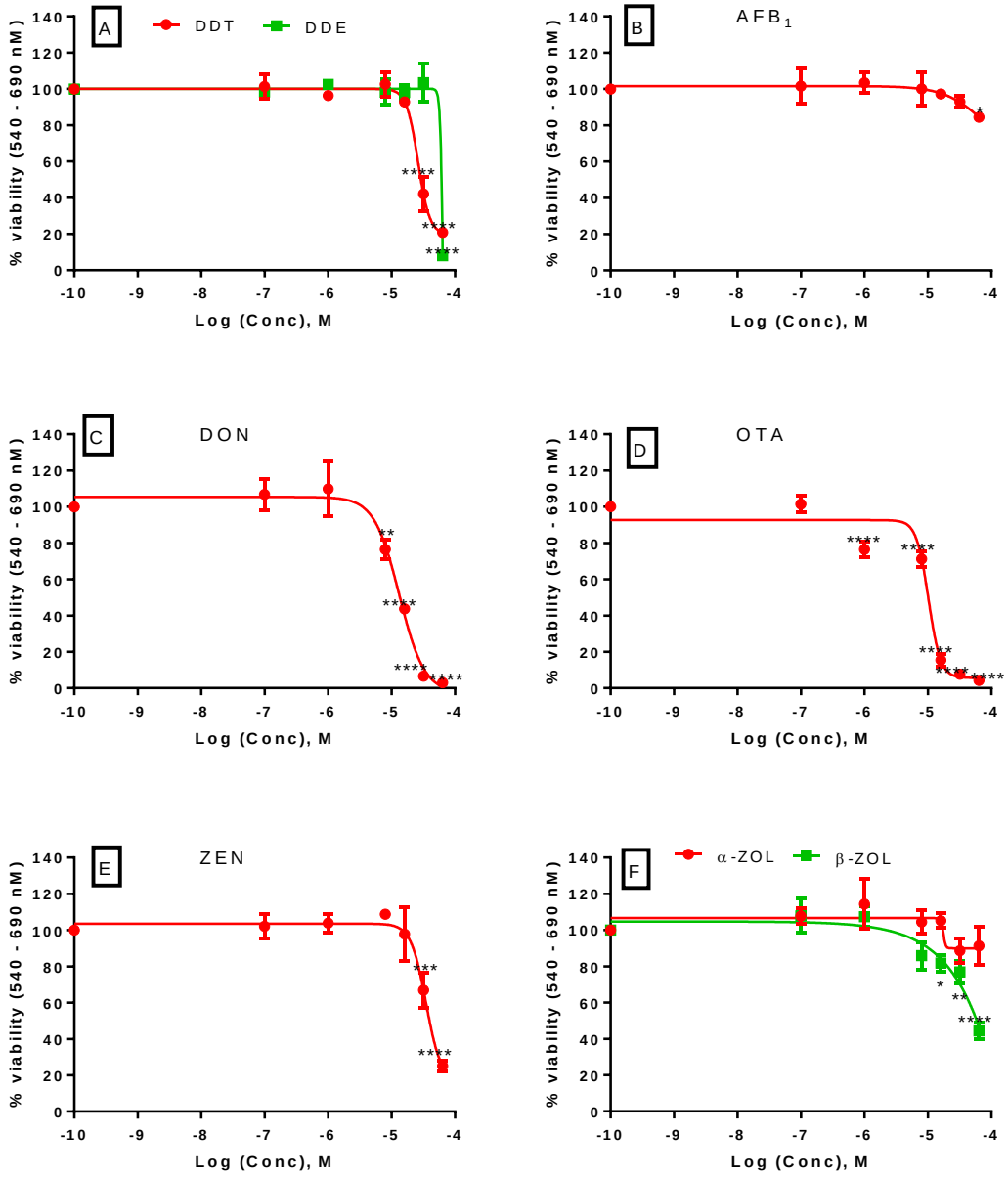

Fig. 5.1: Dose-response curve of the cell viability results using MTT for BeWo placental cells exposed to graded concentrations of mycotoxins and pesticides for 48 hours calculated as a percentage of 0.1% DMSO vehicle control. Each value on the graph is the mean of three independent experiments with three technical replicates each and error bars show the standard deviation (SD) of three biological replicates. Data were analysed using one-way ANOVA with Dunnett's test method to correct for multiple

comparison. $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***) and $P \leq 0.0001$ (****) represent significant effects.

5.2.2 Effects of binary combinations of *p,p′*-DDT and *p,p′*-DDE and/or mycotoxins on BeWo cell viability

The cytotoxic effects of the combinations of mycotoxins with *p,p′*-DDT or *p,p′*-DDE are shown in **Fig. 5.2 and Fig. 5.3**. Binary mixtures of *p,p′*-DDT and AFB_1 produced cytotoxic effects which were similar to that produced by *p,p′*-DDT alone (**Fig. 5.2A**). Combination of *p,p′*-DDT with DON or OTA caused a significant dose-dependent decrease ($P \leq 0.0001$) in BeWo cell viability and these co-treatments enhanced the cytotoxicity of *p,p′*-DDT (**Fig. 5.2B and 5.2C**). In addition, the cytotoxicity of ZEN and its metabolites were amplified by the presence of *p,p′*-DDT (**Fig 5.2D – 5.2F**). In **Fig. 5.3**, the cytotoxicity of mycotoxins were enhanced by the addition of *p,p′*-DDE.

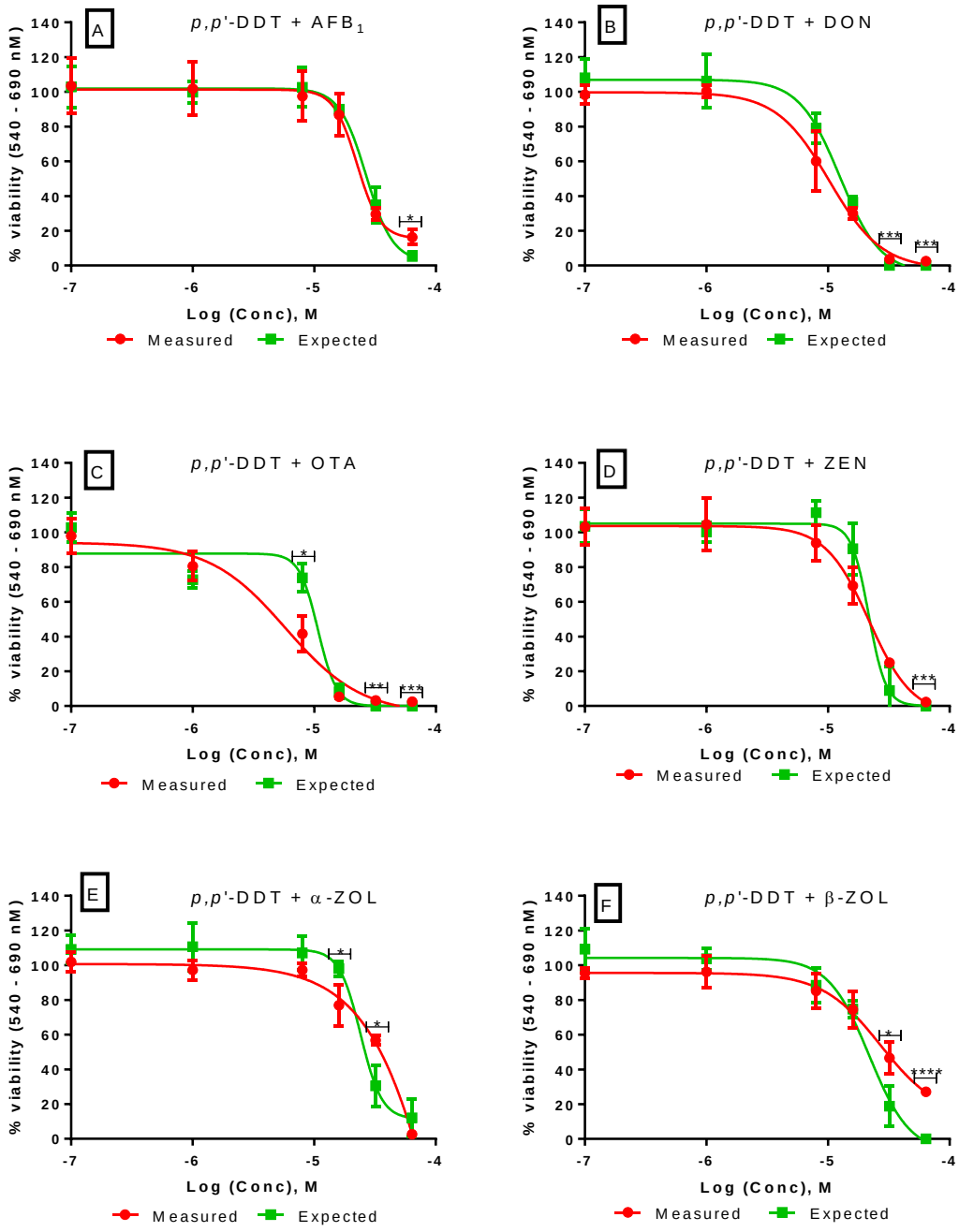

Fig. 5.2: Measured and expected cell viability results using MTT for BeWo placental cells exposed to *p,p'*-DDT in combination with either AFB_1, DON, OTA, ZEN, α-ZOL or β-ZOL for 48 hours calculated as a percentage of 0.2% DMSO vehicle control. Each value on the graph is the mean of three independent experiments with three technical replicates each and error bars show the standard deviation (SD) of three biological replicates. Data were analysed using multiple t-test with Holm-Šídák test method to correct for multiple comparison. $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****) represent significant deviation from additive effects.

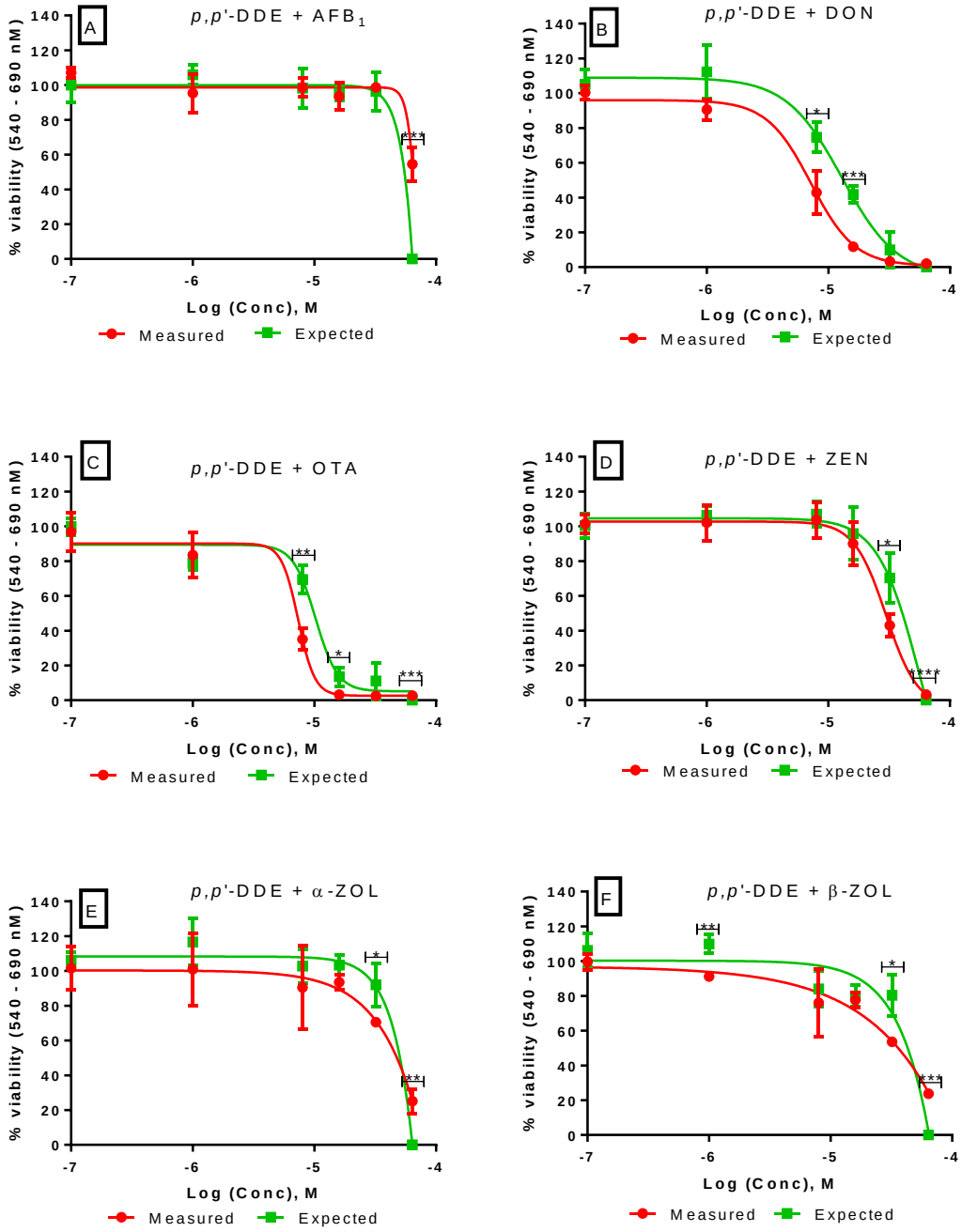

Fig. 5.3: Measured and expected cell viability results using MTT for BeWo placental cells exposed to *p,p'*-DDE in combination with either AFB_1, DON, OTA, ZEN, α-ZOL or β-ZOL for 48 hours calculated as a percentage of 0.2% DMSO vehicle control. Each value on the graph is the mean of three independent experiments with three technical replicates each and error bars show the standard deviation (SD) of three biological replicates. Data were analysed using multiple t-test with Holm-Šídák test method to correct for multiple comparison. $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****) represent significant deviation from additive effects.

5.2.3 Effects of binary mycotoxin mixtures on BeWo placental cell viability

AFB_1 did not induce significant cytotoxicity up to 32 µM when cells were treated alone with this compound (**Fig. 5.1B**), and the cytotoxic effects induced by the combination of AFB_1 with DON or OTA was not different from the effect exhibited by DON or OTA, alone (**Fig. 5.4B & 5.4C**). However, cells exposed to ZEN, α-ZOL or β-ZOL were less sensitive when combined with AFB_1 (**Fig. 5.4D – 5.4F**). Noteworthy is that mixtures of ZEN, α-ZOL or β-ZOL with DON inhibited the cytotoxicity induced by DON, alone (**Fig. 5.5B – 5.5D**). BeWo cells exposed to OTA + α-ZOL, ZEN + α-ZOL, ZEN + β-ZOL and α-ZOL + β-ZOL were less susceptible, especially at 64 µM combinations whereas OTA + β-ZOL was more cytotoxic to the cells than each of the mycotoxins alone (**Fig. 5.6**).

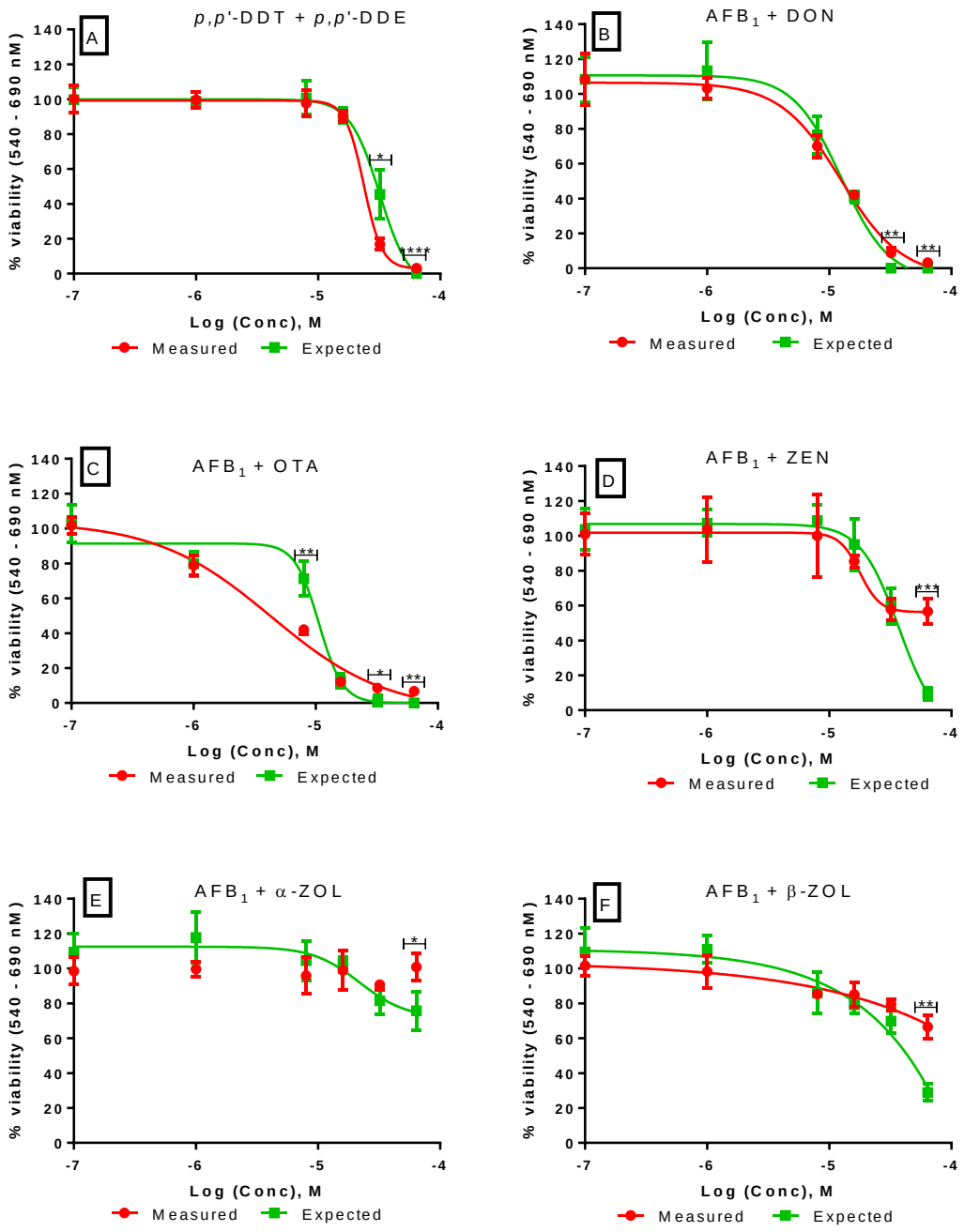

Fig. 5.4: Measured and expected cell viability results using MTT for BeWo placental cells exposed to chemically-defined binary combinations of *p,p'*-DDT and *p,p'*-DDE metabolite (A), and AFB_1 with other mycotoxins (B – F) for 48 hours calculated as a percentage of 0.2% DMSO vehicle control. Each value on the graph is the mean of three independent experiments with three technical replicates and error bars show the standard deviation (SD) of three biological replicates. Data were analysed using multiple t-test with Holm-Šídák test method to correct for multiple comparison. $p \leq 0.05$

(*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****) represent significant deviation from additive effects.

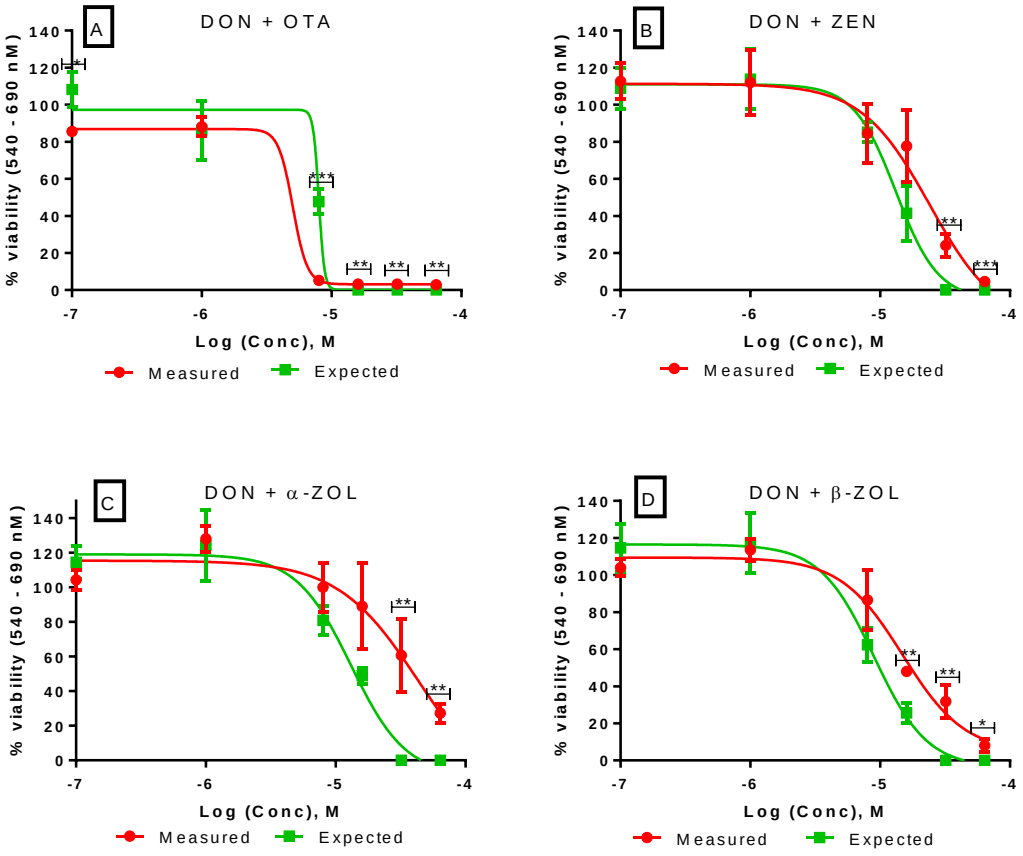

Fig. 5.5: Measured and expected cell viability results using MTT for BeWo placental cells exposed to chemically-defined binary combinations of DON with either OTA, ZEN, α-ZOL or β-ZOL for 48 hours calculated as a percentage of 0.2% DMSO vehicle control. Each value on the graph is the mean of three independent experiments with three technical replicates each and error bars show the standard deviation (SD) of three biological replicates. Data were analysed using multiple t-test with Holm-Šídák test method to correct for multiple comparison. $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****) represent significant deviation from additive effects.

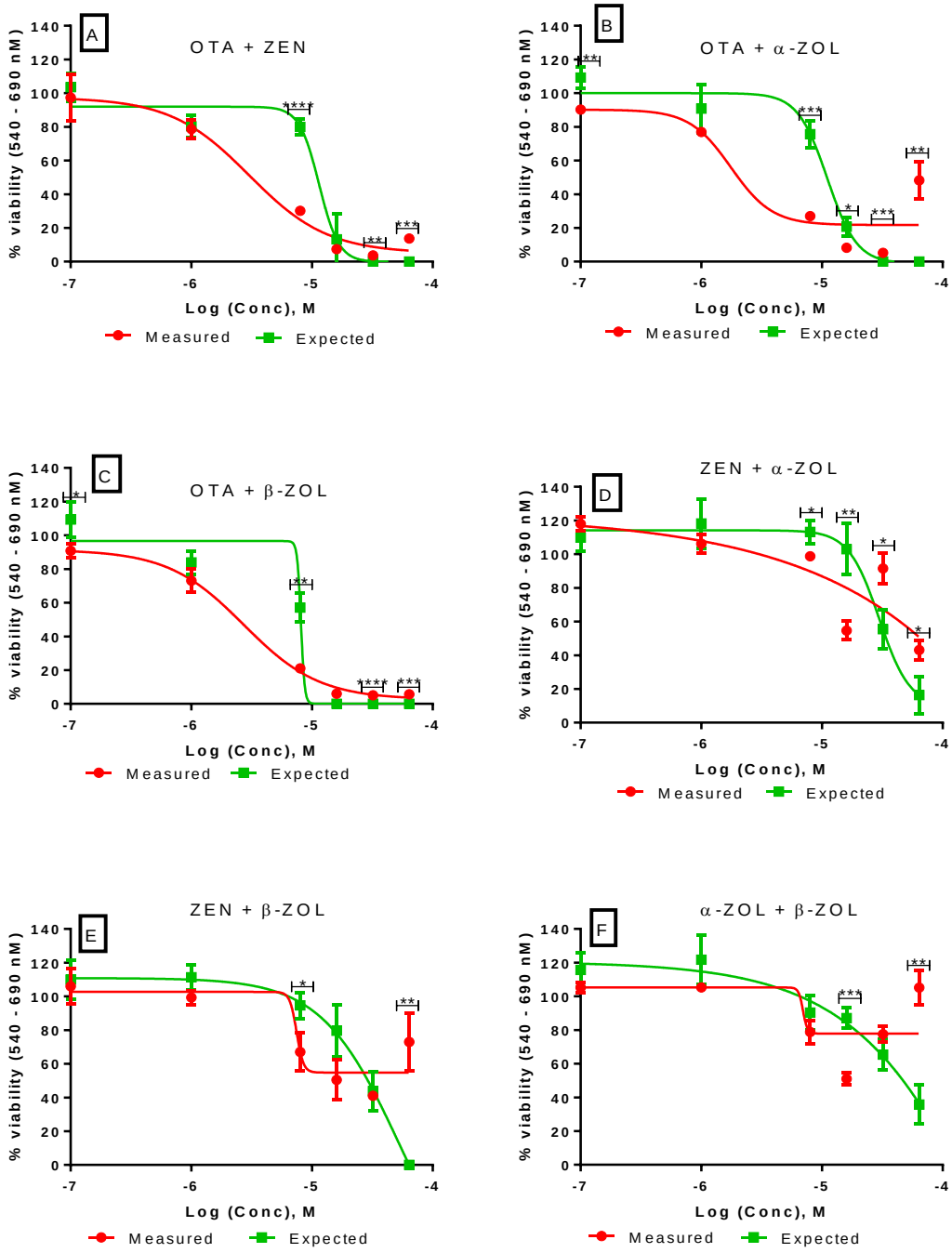

Fig. 5.6: Measured and expected cell viability results using MTT for BeWo cells exposed to chemically-defined binary combinations of OTA with either ZEN, α-ZOL or β-ZOL (A - C), and ZEN with its metabolites (D - F) for 48 hours calculated as a percentage of 0.2% DMSO vehicle control. Each value on the graph is the mean of three independent experiments with three technical replicates each and error bars show the standard deviation (SD) of three biological replicates. Data were analysed using multiple t-test with Holm-Šídák test method to correct for multiple comparison. $p \leq 0.05$

(*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****) represent significant deviation from additive effects.

5.2.4 Interactive effects of binary mycotoxin and pesticide mixtures on BeWo placental cell viability

The interactive cytotoxic effects of binary combinations of mycotoxins and pesticides were determined based on the comparison of theoretical expected cell viability values from single treatments compared with the observed cell viability values of co-exposure experiment using a multiple t-test with Holm-Sidak test to correct for multiple comparison (**Fig. 5.2 - Fig. 5.6**; **Table 5.1**). It was observed that the combination of *p,p'*-DDT with OTA, DON, ZEN, α-ZOL or β-ZOL showed additive and synergistic interactive effects on BeWo cell viability at lower doses and antagonistc effects at higher concentration (**Fig. 5.2B - Fig. 5.2F**). Similarly, *p,p'*-DDE + OTA, *p,p'*-DDE + DON and *p,p'*-DDE + β-ZOL exhibited synergistic cytotoxic effects in almost all the concentrations tested, but combinatory effects were antagonistic at 64 μM (**Fig. 5.3A - Fig. 5.3F**). Also worthy of note, mixtures of OTA + ZEN, OTA + α-ZOL, OTA + β-ZOL, ZEN + α-ZOL, ZEN + β-ZOL and α-ZOL + β-ZOL produced synergistic cytotoxic effects at ≤ 16 μM **(Fig. 5.6**). In general, most of the mycotoxin and pesticide mixtures produced synergistic or additive interaction at lower concentrations, but showed antagonistic interactive effects at concentrations above 16 μM (**Table 5.1**).

Table 5.1: Summary of the interactive cytotoxic effects of binary mixtures of mycotoxins and pesticides on BeWo cell line

Toxin combinations	**Dose combinations (µM) and interaction effects of binary mixtures**					
	0.1: 0.1	**1 : 1**	**8 : 8**	**16 : 16**	**32 : 32**	**64 : 64**
p,p′-DDT + *p,p′*-DDE					p = 0.0268[a]	p < 0.0001[b]
					Diff. = -28.55	Diff. = 3.11
p,p′-DDT + AFB1						p = 0.0189[b]
						Diff. = 11.08
p,p′-DDT + DON					p = 0.0003882[b]	p = 0.000964[b]
					Diff. = 3.67	Diff. = 2.56
p,p′-DDT + OTA			p = 0.01271[a]		p = 0.002651[b]	p = 0.000964[b]
			Diff. = -32.22		Diff. = 3.22	Diff. = 2.56
p,p′-DDT + ZEN						p = 0.000562[b]
						Diff. = 2.22
p,p′-DDT + α-ZOL				p = 0.04576[a]	p = 0.01970[b]	
				Diff. = 21.11	Diff. = 26.22	

Toxin combinations	Dose combinations (μM) and interaction effects of binary mixtures					
	0.1: 0.1	**1 : 1**	**8 : 8**	**16 : 16**	**32 : 32**	**64 : 64**
p,p′-DDT + β-ZOL					p = 0.02982[b]	p < 0.0001[b]
					Diff. = 27.67	Diff. = 27.11
p,p′-DDE + AFB_1						p = 0.000627[b]
						Diff. = 54.56
p,p′-DDE + DON			p = 0.02264[a]	p = 0.0004634[a]		
			Diff. = -31.78	Diff. = -30.00		
p,p′-DDE + OTA			p = 0.004374[a]	p = 0.02994[a]		p = 0.0001573[b]
			Diff. = -34.33	Diff. = -10.33		Diff. = 2.67
p,p′-DDE + ZEN					p = 0.03883[a]	p < 0.0001[b]
					Diff. = 27.33	Diff. = 3.22
p,p′-DDE + α-ZOL					p = 0.04094[a]	p = 0.003264[b]
					Diff. = -21.44	Diff. = 25.11
p,p′-DDE + β-ZOL		p = 0.006141[a]			p = 0.01895[a]	p = 0.0001068[b]
		Diff. = -18.67			Diff. = -26.77	Diff. = 23.78

Toxin combinations	Dose combinations (µM) and interaction effects of binary mixtures					
	0.1: 0.1	**1 : 1**	**8 : 8**	**16 : 16**	**32 : 32**	**64 : 64**
AFB_1 + DON					p = 0.005137[b]	p = 0.001992[b]
					Diff. = 8.83	Diff. = 3.17
AFB_1 + OTA			p = 0.00816[a]		p = 0.02249[b]	p = 0.001701[b]
			Diff. = -29.22		Diff. = 8.02	Diff. = 6.75
AFB_1 + ZEN						p = 0.0005827[b]
						Diff. = 47.11
AFB_1 + α-ZOL						p = 0.03258[b]
						Diff. = 25.05
AFB_1 + β-ZOL						p = 0.001450[b]
						Diff. = 37.61
DON + OTA	p = 0.01687[a]		p = 0.0004134[a]	p = 0.001297[b]	p = 0.001297[b]	p = 0.003967[b]
	Diff. = -22.66		Diff. = -42.45	Diff. = 3.22	Diff. = 3.22	Diff. = 2.89
DON + ZEN					P = 0.002309[b]	P = 0.0007868[b]
					Diff. = 24.11	Diff. = 4.67

Toxin combinations	Dose combinations (µM) and interaction effects of binary mixtures					
	0.1: 0.1	**1 : 1**	**8 : 8**	**16 : 16**	**32 : 32**	**64 : 64**
DON + α-ZOL					p = 0.007385[b]	p = 0.001006[b]
					Diff. = 60.67	Diff. = 27.33
DON + β -ZOL				p = 0.002416[b]	p = 0.003442[b]	p = 0.0136[b]
				Diff. = 22.44	Diff. = 31.78	Diff. = 8.11
OTA + ZEN			p < 0.0001[a]		p = 0.001971[b]	p = 0.0008113[b]
			Diff. = -49.89		Diff. = 3.67	Diff. = 13.67
OTA + α-ZOL	p = 0.00745[a]		p = 0.0004609[a]	P = 0.01721[a]	p = 0.0003001[b]	p = 0.001551[b]
	Diff. = -19.00		Diff. = -48.67	Diff. = -12.44	Diff. = 5.22	Diff. = 48.22
OTA + β -ZOL	p = 0.04595[a]		p = 0.001996[a]		p < 0.0001[b]	p = 0.0001239[b]
	Diff. = -18.55		Diff. = -36.11		Diff. = 5.11	Diff. = 5.66
ZEN + α-ZOL			p = 0.02457[a]	p = 0.006535[a]	p = 0.01409[b]	p = 0.02084[b]
			Diff. = -14.44	Diff. = -48.33	Diff. = 36.12	Diff. = 26.67
ZEN + β -ZOL			p = 0.02467[a]			p = 0.001773[b]
			Diff. = -27.67			Diff. = 73.00

Toxin combinations	Dose combinations (µM) and interaction effects of binary mixtures					
	0.1: 0.1	**1 : 1**	**8 : 8**	**16 : 16**	**32 : 32**	**64 : 64**
α-ZOL+ β -ZOL				p = 0.0008840[a]		p = 0.00152[b]
				Diff. = -36.11		Diff. = 69.39

Key: [a]Synergistic = measured cell viability values were significantly below expected values; **[b]Antagonistic =** measured cell viability values were significantly above expected values; **Diff.** = Mean difference

5.2.5 Effects of single mycotoxins and pesticide on progesterone (P4) production

The basal P4 level in the cell media supernatant of BeWo cells exposed to DMSO (0.1%) vehicle control for 48 h was 49.92 ± 6.40 ng/mL whereas this concentration increased to 164.12 ± 33.15 ng/mL (3.3 fold) after treatment with the positive control (8-Br-cAMP: 1 mM). The pesticide *p,p'*-DDT reduced P4 secretion by 46.0% and 64.6 % at 8 µM and 16 µM, respectively (**Fig. 5.7A**). Among ZEN and its metabolites, ZEN and α-ZOL had no significant effect on P4 release whereas β-ZOL significantly ($p \leq 0.001$) increased P4 level at 16 µM (74.93 ± 28.33 ng/mL) compared to vehicle control (**Figs. 5.7B - 5.7D**). DON and OTA decreased P4 level in dose dependently, but this occurred at concentrations which had cytotoxic effects on BeWo cell viability (**Fig. 5.1; Fig. 5.7E & 5.7F**).

5.2.6 Effects of single mycotoxins and pesticide *p,p'*-DDT on oestradiol (E2) production

The basal E2 level in the residual cell culture medium of BeWo cells treated with DMSO (0.1%) was 43.00 ± 11.26 pg/mL after 48 h treatment. Treatment of BeWo cells with 1 mM of Br-cAMP strongly stimulated E2 secretion (476.52 ± 47.54 pg/mL). Exposure to ZEN and its metabolites significantly ($p \leq 0.01$) elevated E2 release in cell media supernatant in dose dependent manner (**Figs. 5.8B - 5.8D**). The highest concentration of E2 occurred at 16 µM of α-ZOL (113.82 ± 2.90 pg/mL), followed by β-ZOL (109.13 ± 31.90 pg/mL) whereas the parent compound ZEN induced the lowest level of E2 at 16 µM (75.46 ± 4.19 pg/mL). Although OTA and DON significantly inhibited E2 secretion, particularly at 16 µM, but this concentration had cytotoxic effects on BeWo cell viability (**Fig. 5.1; Fig. 5.8E & 5.8F**)

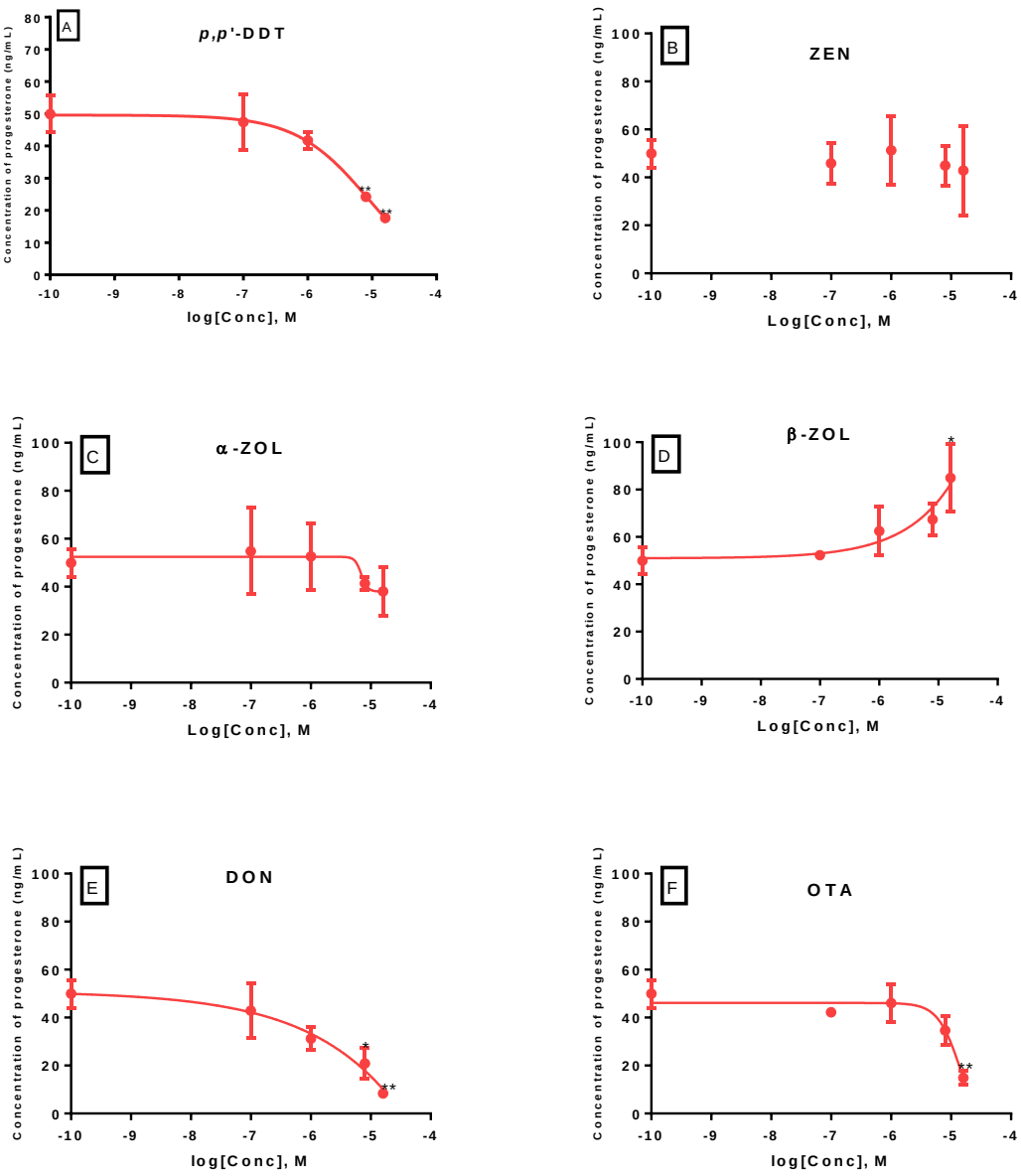

Fig. 5.7: The effects of single mycotoxins and pesticides exposure on the progesterone (P4) production in BeWo placental cells. Cells were exposed to the compounds for 48 h and cell media supernatants were collected for P4 analysis using enzyme-linked immunosorbent assay (ELISA). Each value on the graph is the mean of two independent experiments with three technical replicates each and error bars show the standard deviation (SD) of two biological replicates. Data were analysed by comparing treatments and vehicle control using one-way ANOVA and corrected for multiple comparison with Dunnett's test. $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****) represent significant effects. DON: deoxynivalenol; OTA: ochratoxin A; α-ZOL: alpha-zearalenol; β-ZOL: beta-zearalenol; *p,p'*-DDT: 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane

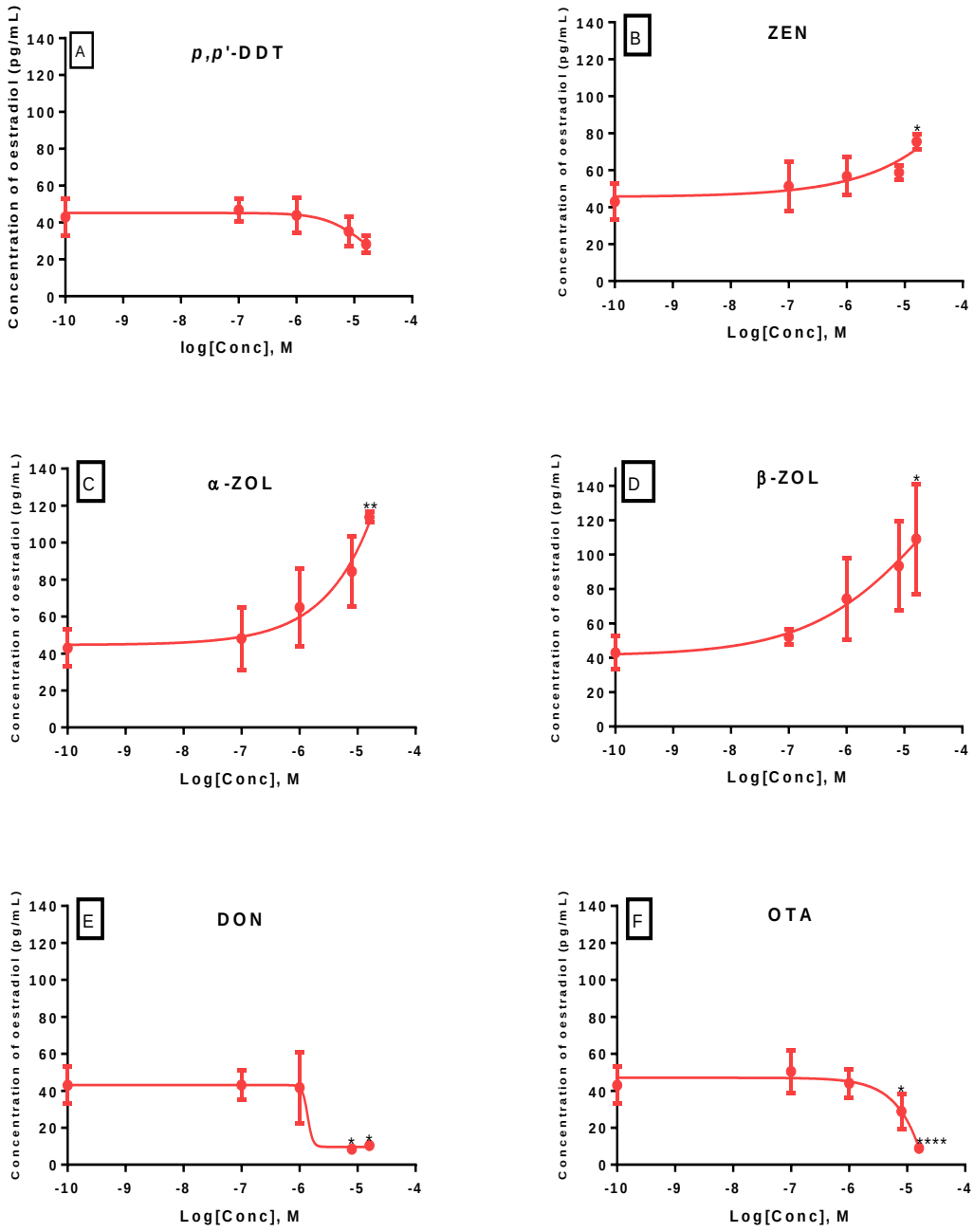

Fig. 5.8: The effects of single mycotoxins and pesticides exposure on the oestradiol (E2) production in BeWo placental cells. Cells were exposed to the compounds for 48 h and cell media supernatants were collected for E2 analysis using enzyme-linked immunosorbent assay (ELISA). Each value on the graph is the mean of two independent experiments with three technical replicates each and error bars show the standard deviation (SD) of two biological replicates. Data were analysed by comparing treatments and vehicle control using one-way ANOVA and corrected for multiple comparison with Dunnett's test. $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****) represent significant effects.

5.2.7 Effects of single mycotoxins and pesticide *p,p'*-DDT on the production of beta-human chorionic gonadotropin (β-hCG) in BeWo placental cells

The basal β-hCG level in the residual cell culture medium of BeWo cells treated with DMSO (0.1%) was 290.02 ± 19.98 mIU/mL after 48 h treatment. Treatment of BeWo cells with 1 mM of 8-Br-cAMP stimulated β-hCG secretion by 22.5 fold (6531 ± 198.87 mIU/mL). All the mycotoxins and p,p-DDT strongly inhibited ($p \leq 0.001$) the production of β-hCG in BeWo cells (**Figs 5.9A - 5.9F**). However, the level of β-hCG hormone gradually increased after treatment with *p,p'*-DDT (8 µM and 16µM), although these could not reach the level observed in the vehicle control (**Fig. 5.9A**).

5.2.8 Effects of binary mixtures of mycotoxins and pesticide *p,p'*-DDT on BeWo placental cell hormone production

The equimolar binary mixtures of mycotoxins and pesticide concentrations showing at least 80% cell viability (**Fig. 5.2 - 5.6**) were selected for further analysis on their effect on BeWo cell hormone production. Treatments of BeWo cells with *p,p'*-DDT/α-ZOL, *p,p'*-DDT/β-ZOL, DON/α-ZOL and DON/β-ZOL reduced E2 secretion in cell media supernatant at low dose combinations (0.1 µM or 1 µM), and these interactive effects were synergistic (**Fig. 5.10**). Binary combinations *p,p'*-DDT/ZEN and DON/ZEN inhibited E2 production in dose dependent relationship compared to the DMSO control (**Fig. 5.10A & 5.10D**), indicating that p,p-DDT or DON had interactive activity on the stimulatory effect observed by ZEN, alone. In addition, ZEN/α-ZOL or α-ZOL/β-ZOL induced E2 secretion (1.6 - 2.0 fold) and these mixture interactions were synergistic (**Fig. 5.10G & Fig. 5.10H**).

Binary combination of DDT or DON with ZEN, α-ZOL or β-ZOL caused inhibition of P4 release in BeWo cell media supernatant and these occurred with respect to increase in toxin concentration whereas ZEN/α-ZOL and α-ZOL/β-ZOL had no significant effect on P4 production (**Fig. 5.11**). With regard to β-hCG production in BeWo cells, *p,p'*-DDT/ZEN, *p,p'*-DDT/β-ZOL, ZEN/α-ZOL, α-ZOL/β-ZOL stimulated β-hCG level at 0.1 µM and 1 µM dose combinations and these were higher than the concentrations of β-hCG observed after exposure of BeWo cells to these toxins, alone (**Fig. 5.12A, 5.12G & 5.12H**). Binary combinations of DON with ZEN, α-ZOL or β-ZOL at equimolar concentrations of 0.1 µM stimulated β-hCG release higher than the one observed for each toxin alone, but inhibited β-hCG secretion by BeWo cells at 1 µM and the level of

β-hCG was completely inhibited by these mycotoxin combinations at 8 µM dose combinations (**Fig. 5.12D - 5.12F**).

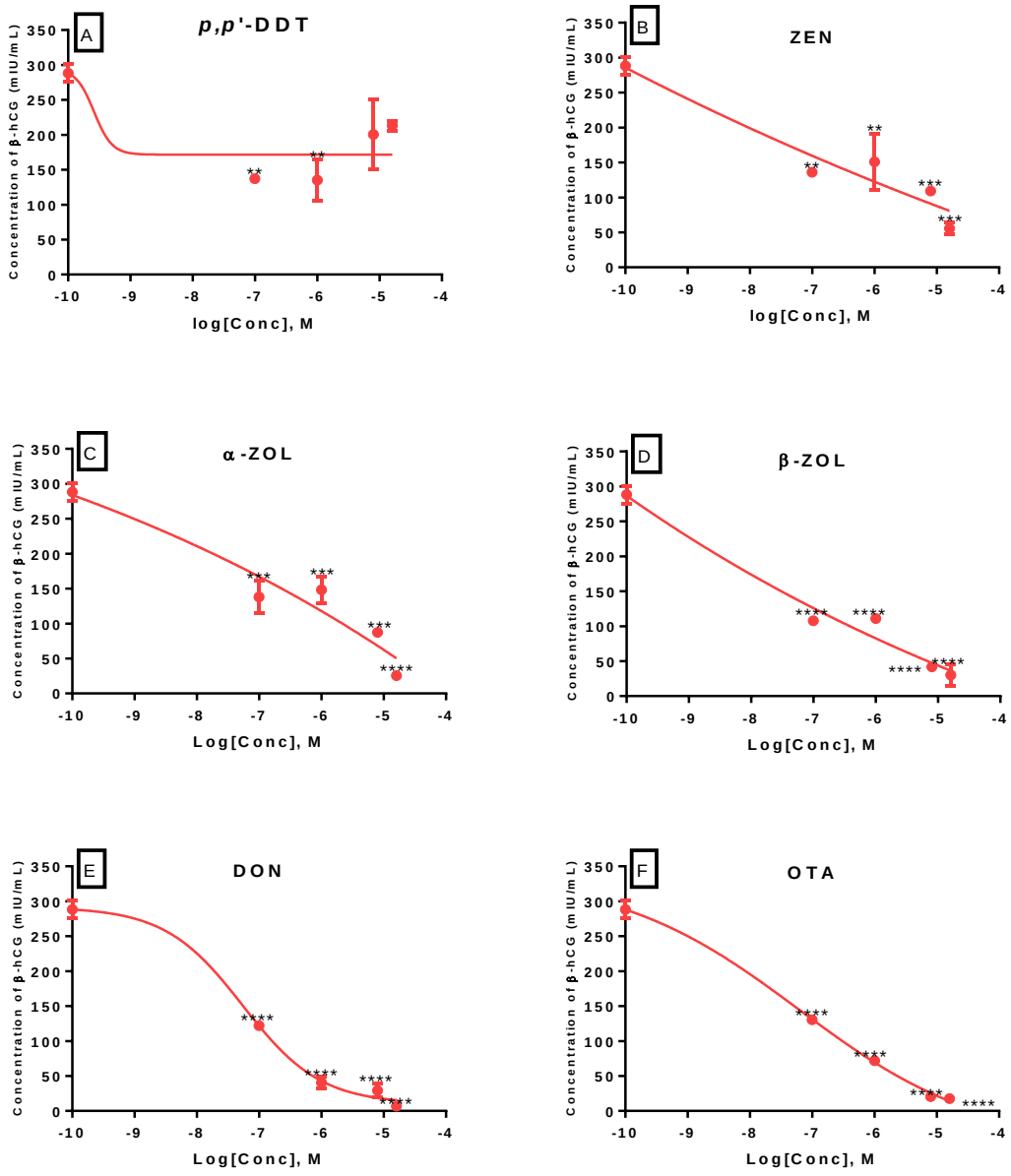

Fig. 5.9: The effects of single mycotoxins and pesticides exposure on the beta-human chorionic gonadotrophin (β-hCG) production in BeWo placental cells. Cells were exposed to the compounds for 48 h and cell media supernatants were collected for β-hCG analysis using enzyme-linked immunosorbent assay (ELISA). Each value on the graph is the mean of two independent experiments with three technical replicates each and error bars show the standard deviation (SD) of two biological replicates. Data were analysed by comparing treatments and vehicle control using one-way ANOVA and corrected for multiple comparison with Dunnett's test. $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****) represent significant effects.

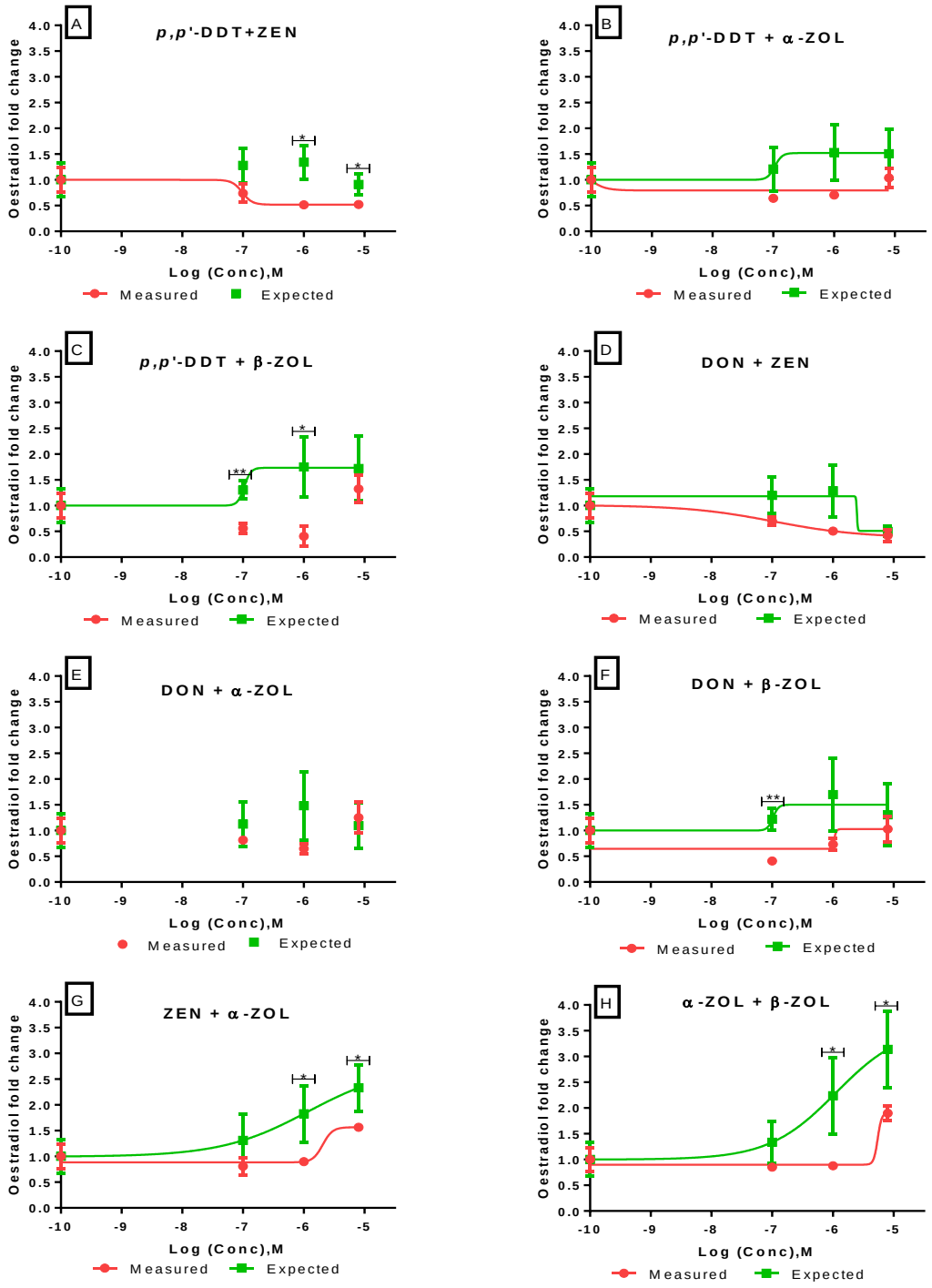

Fig. 5.10: Measured amd expected fold change in oestradiol (E2) production in BeWo placental cells cells exposed to binary mixtures of *p,p'*-DDT or DON with ZEN and its metabolites. Test substances were assayed in triplicate in two independent experiments. Data were analysed with multiple t-test and corrected for multiple comparson using the Holm-Šídák test method. Error bars represent standard deviation of three biological replicates. $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****) represent significant deviation from additive effects.

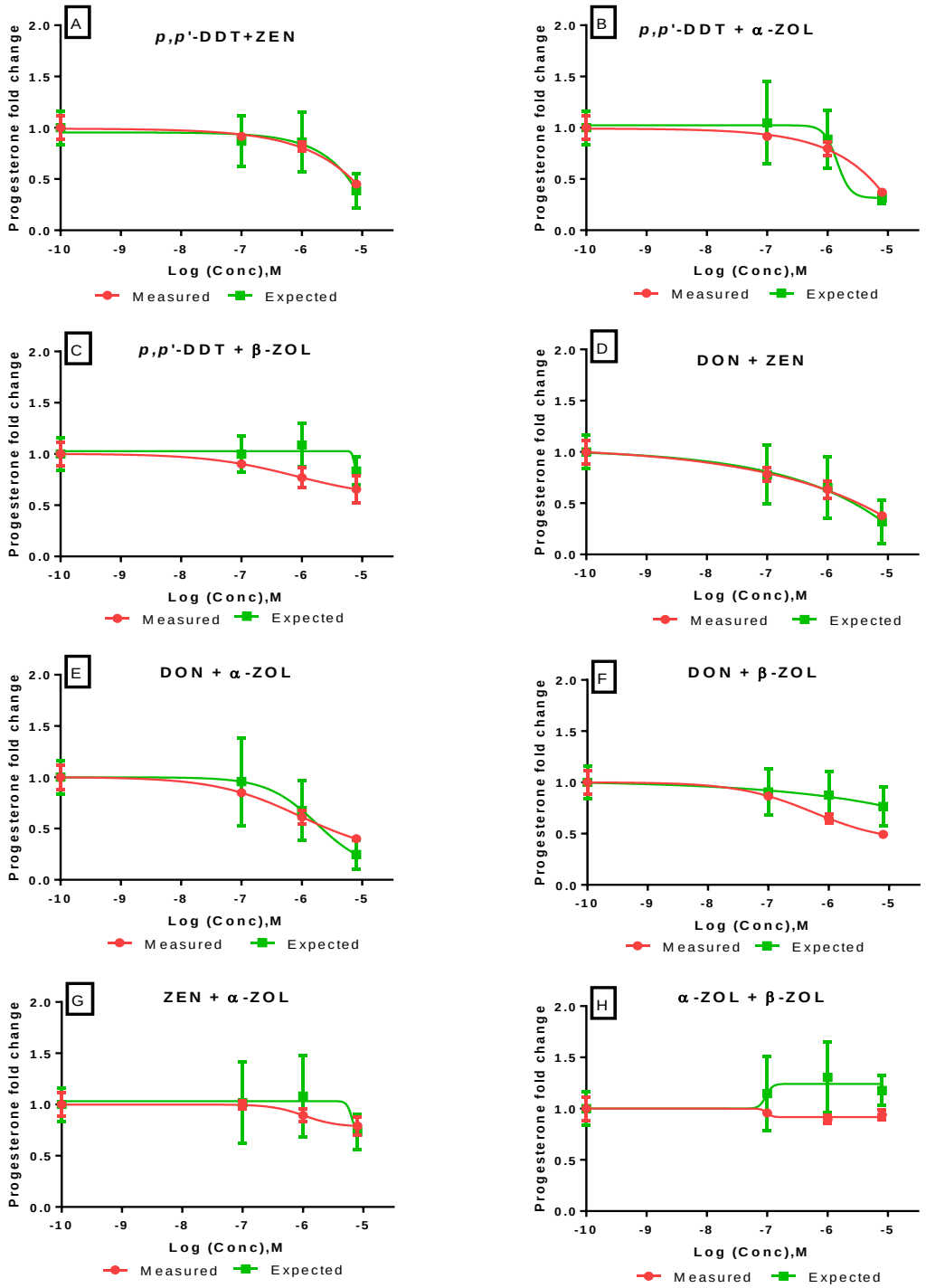

Fig. 5.11: Measured amd expected fold change in progesterone (P4) production in BeWo placental cells cells exposed to binary mixtures of *p,p'*-DDT or DON with ZEN and its metabolites. Test substances were assayed in triplicate in two independent experiments. Data were analysed with multiple t-test and corrected for multiple comparson using the Holm-Šídák test method. Error bars represent standard deviation of two biological replicates. $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****) represent significant deviation from additive effects.

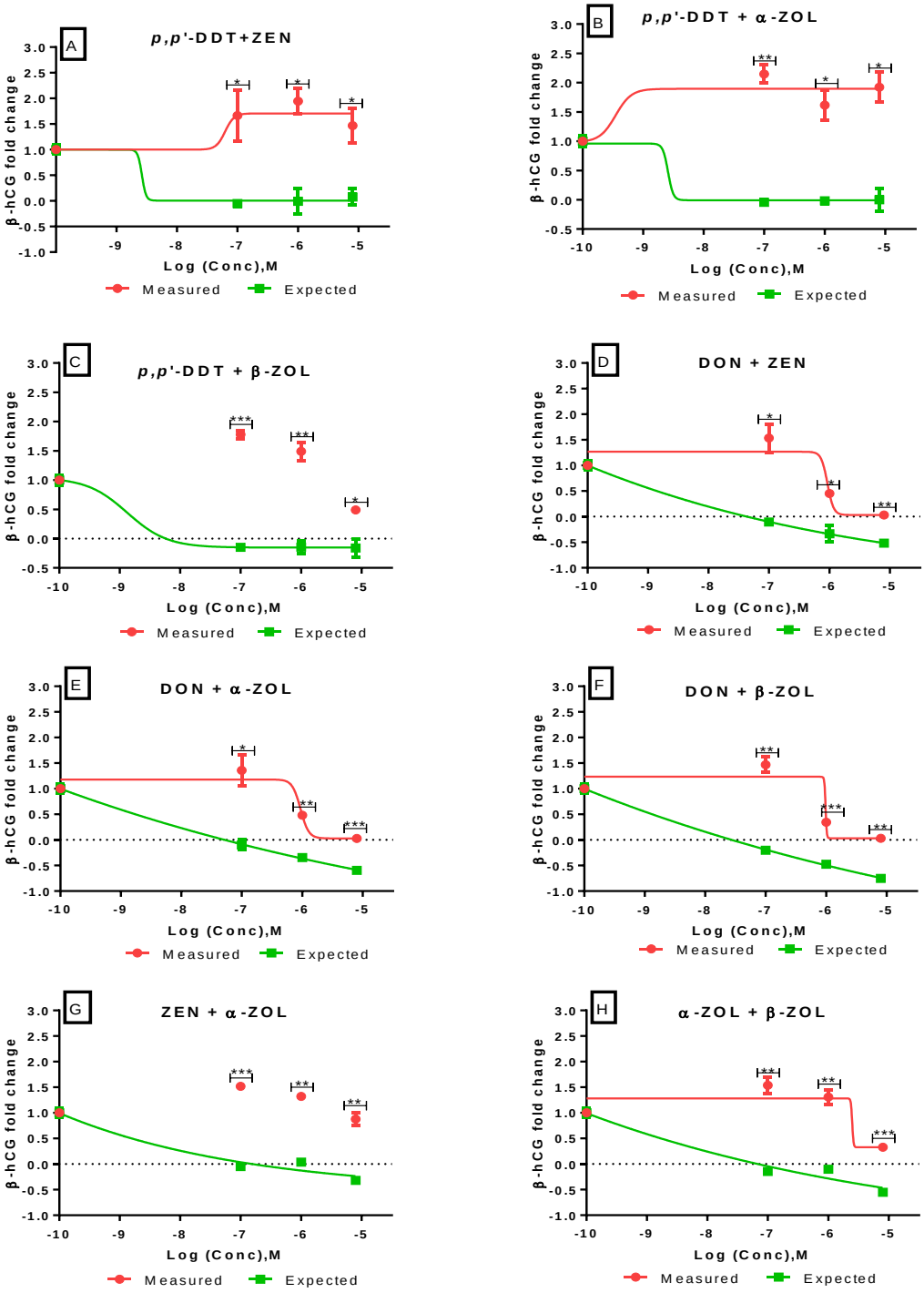

Fig. 5.12: Measured amd expected fold change in beta-human chorionic gonadotropin (β-hCG) production in BeWo placental cells cells exposed to binary mixtures of *p,p'*-DDT or DON with ZEN and its metabolites. Test substances were assayed in triplicate in two independent experiments. Data were analysed with multiple t-test and corrected for multiple comparson using the Holm-Šídák test method. Error bars represent standard deviation of two biological replicates. $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****) represent significant deviation from additive effects

5.2.9 Effects of ZEN and *p,p′*-DDT in single or mixtures on mRNA expression in BeWo cells

p,p′-DDT or *p,p′-DDT*/ZEN at 0.1 µM and 8 µM dose combinations had no significant effect on the mRNA abundance of *ABCC1* and *ABCC2* whereas ZEN at 8.0 µM significantly ($p = 0.004324$) reduced *ABCC2* mRNA level ((**Fig. 5.13A**). On the other hand, *p,p′*-DDT or *p,p′-DDT*/ZEN significantly ($p < 0.05$) upregulated the mRNA expression of *CYP11A1* especially at 8.0 µM (**Fig. 5.13A**). ZEN had no effect on the mRNA expression of ESR1, PGR, AR and GPER1 in BeWo cells at 0.01 µM exposure, but significantly reduced the gene expression level of PGR and AR (**Fig. 5.13B**). Also worthy of note is that *p,p′*-DDT modulated the expression of ESR (**Fig. 3.5**). Regarding genes of the IGF-axis, analysis of variance also revealed that *p,p′*-DDT, ZEN or *p,p′*-DDT/ZEN at 0.1 µM and 8 µM upregulated the mRNA expression level of *IGF2BP1*, but only *p,p′*-DDT at 8.0 µM significantly ($p = 0.026$) the level of *IGF2R* mRNA in BeWo cells (**Fig. 5.14A**). ZEN (0.1 µM), *p,p′*-DDT (0.1 µM and 8 µM) and *p,p′*-DDT/ZEN (0.1 µM and 8 µM) also caused a significant increase in the expression of the imprinted genes *PHLDA2* and *MEG3* while *p,p′*-DDT (0.1 µM and 8 µM) and *p,p′*-DDT/ZEN (0.1 µM and 8 µM) caused 2 - 4 fold increase in the mRNA abundance of genes involved in DNA methylation (*MTHFR* and *MBD2*; **Fig. 5.14B**). Among the genes involved in trophoblast syncytialisation and differentiation, invasion and angiogenesis, binary combinations of *p,p′*-DDT and ZEN particularly at 8 µM caused significant upregulation of *LHCGR*, *ERVW-1*, *VEGF*, *MMP2* and *ZFP57* mRNA in BeWo cells treated for 48 h, with mRNA abundance of MMP2 reaching about 10 fold increase (**Fig. 5.15 & Fig. 5.16B**). Interestingly, *p,p′*-DDT and ZEN co-exposure had little or no effect on genes for for the IGF-axis, DNA methylation and genomic imprinting (**Fig, 5.17**) and plancental differentiation and angiogenesis in BeWo cells (**Fig. 18**).

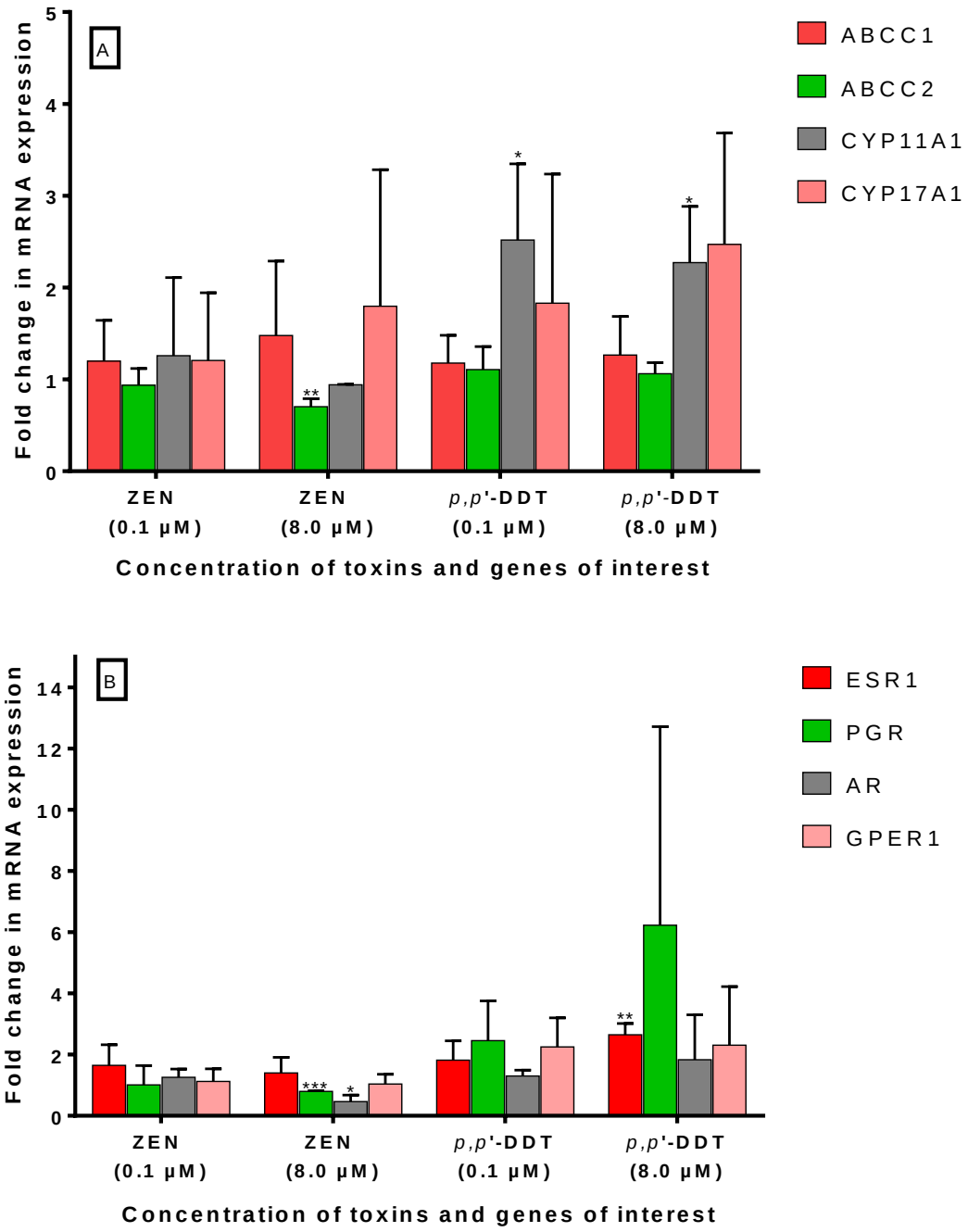

Fig. 5.13: Influence of Zearalenone (ZEN) and 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane (*p,p'*-DDT) on the mRNA expression of ABC transporters and genes for steroidogenesis in BeWo cells using RT-qPCR. The data represent the mean±SD of three biological replicates. Data were analysed using multiple t-test and corrected for multiple comparsion with Holm-Šídák test method. $p \leq 0.05$*, $p \leq 0.01$**, $p \leq 0.001$*** and $p \leq 0.0001$**** represents significant fold change in mRNA expression.

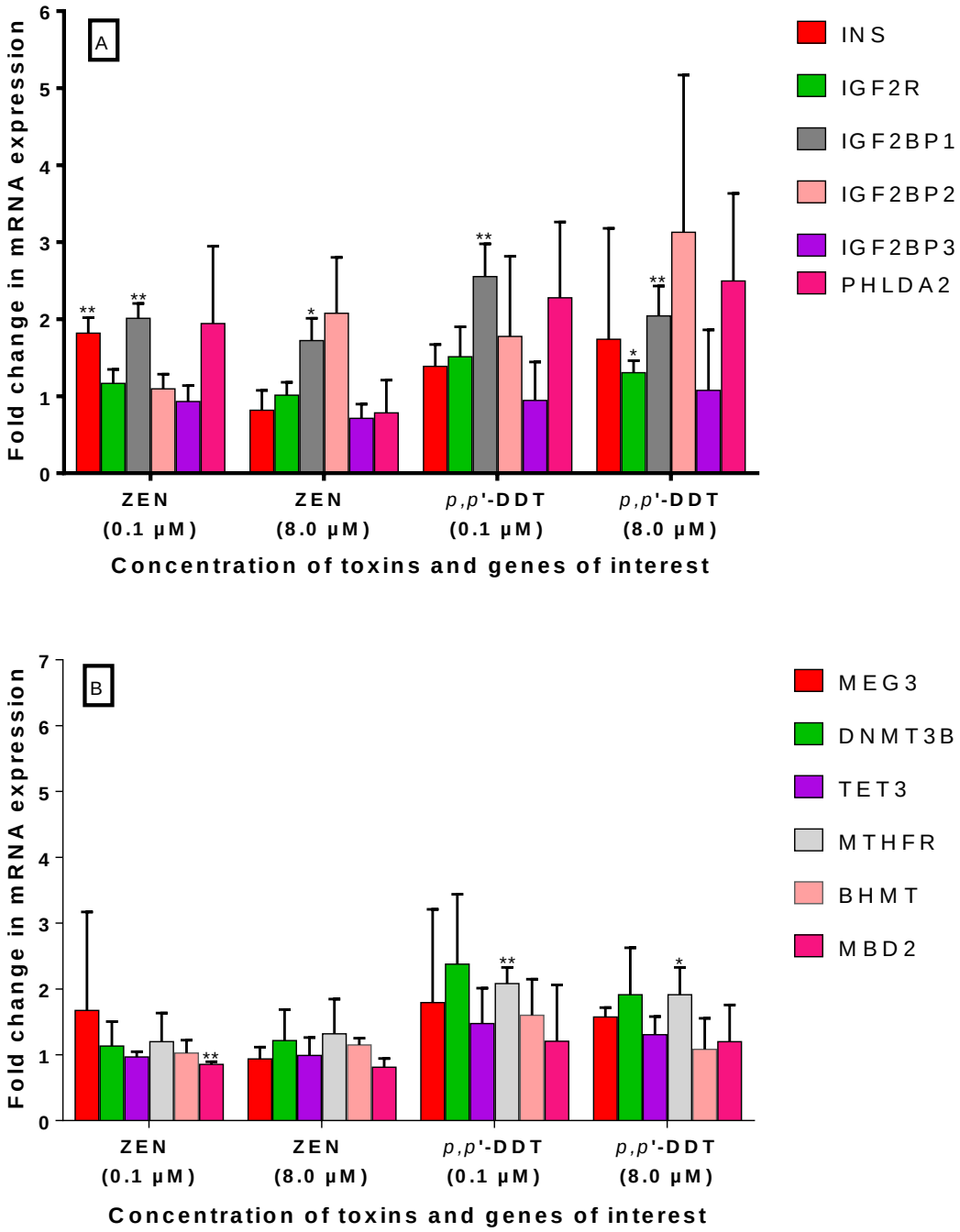

Fig. 5.14: Influence of Zearalenone (ZEN) and 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane (*p,p'*-DDT) on the mRNA expression of genes for the IGF-axis, DNA methylation and genomic imprinting in BeWo cells using RT-qPCR. The data represent the mean±SD of three biological replicates. Data were analysed using multiple t-test and corrected for multiple comparsion with Holm-Šídák test method. $p \leq 0.05$*, $p \leq 0.01$**, $p \leq 0.001$*** and $p \leq 0.0001$**** represents significant fold change in mRNA expression.

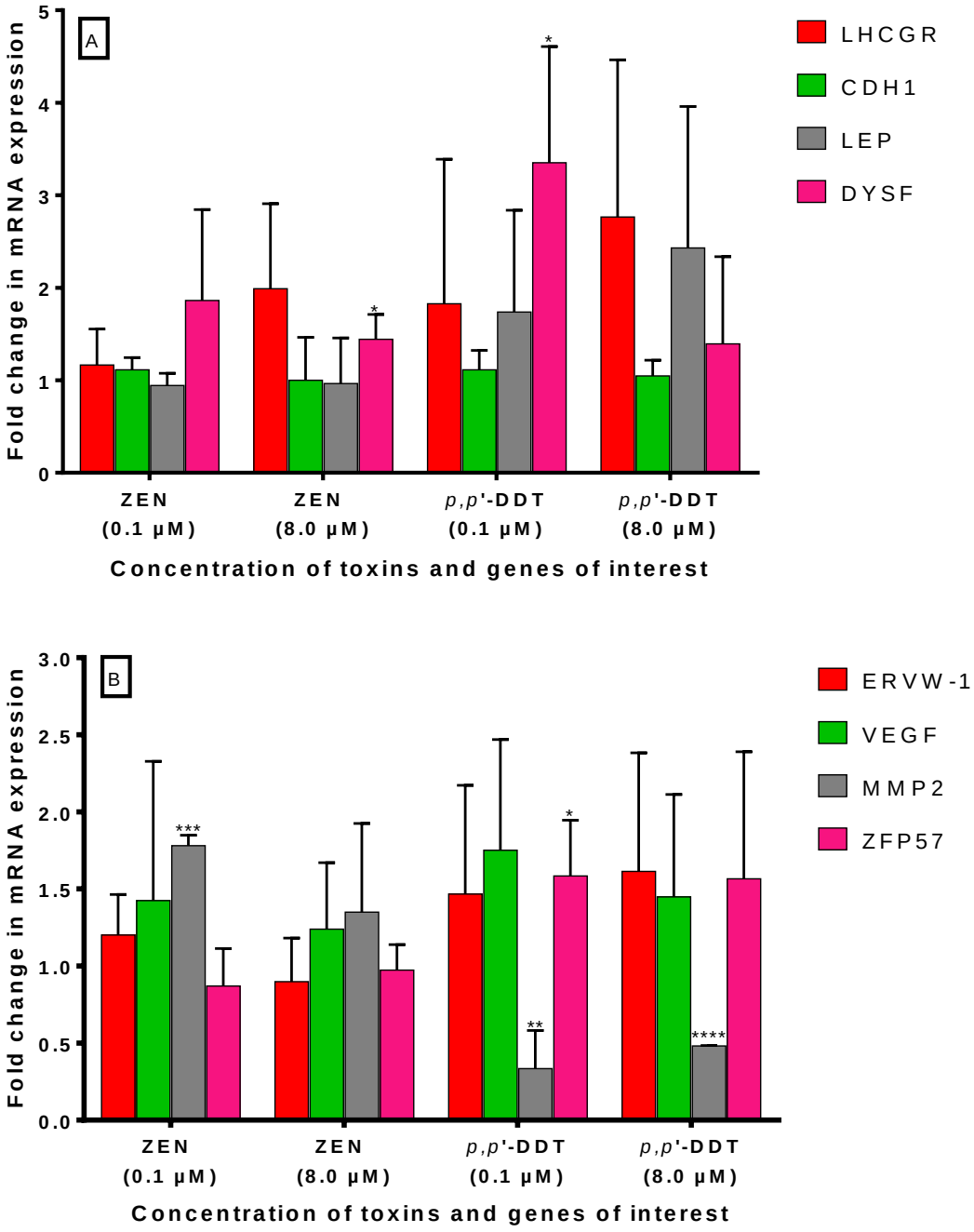

Fig. 5.15: Influence of Zearalenone (ZEN) and 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane (*p,p'*-DDT) on the mRNA expression of genes for plancental differentiation and angiogenesis in BeWo cells using RT-qPCR. The data represent the mean±SD of three biological replicates. Data were analysed using multiple t-test and corrected for multiple comparsion with Holm-Šídák test method. $p \leq 0.05$*, $p \leq 0.01$**, $p \leq 0.001$*** and $p \leq 0.0001$**** represents significant fold change in mRNA expression.

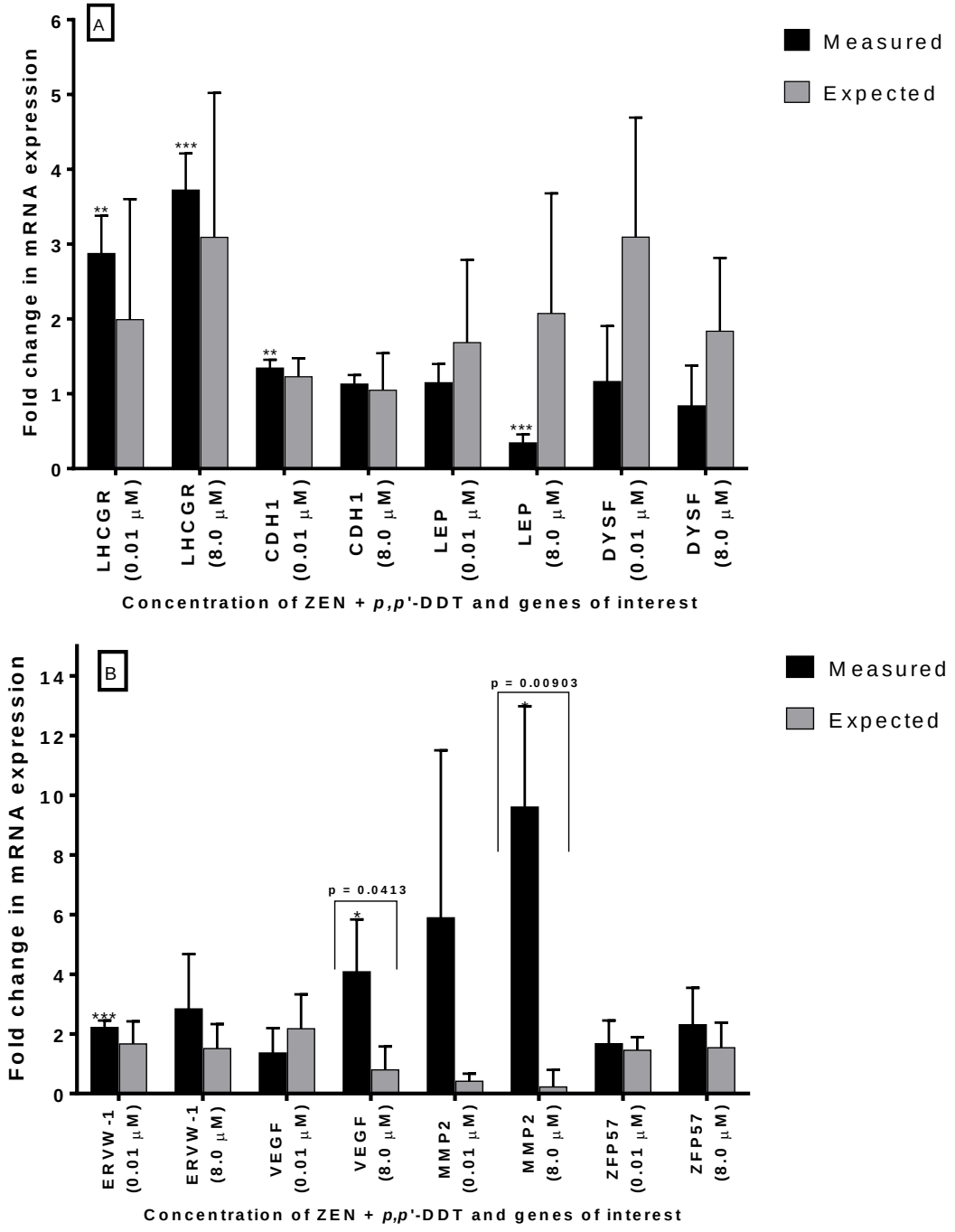

Fig. 5.16: Influence of binary mixtures of zearalenone (ZEN) and 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane (*p,p'*-DDT) on the mRNA expression of ABC transporters and genes for steroidogenesis in BeWo cells using RT-qPCR. The data represent the mean±SD of three biological replicates. Data were analysed using multiple t-test and corrected for multiple comparsion with Holm-Šídák test method. $p \leq 0.05$*, $p \leq 0.01$**, $p \leq 0.001$*** and $p \leq 0.0001$**** represents significant fold change in mRNA expression and deviation from additve effects.

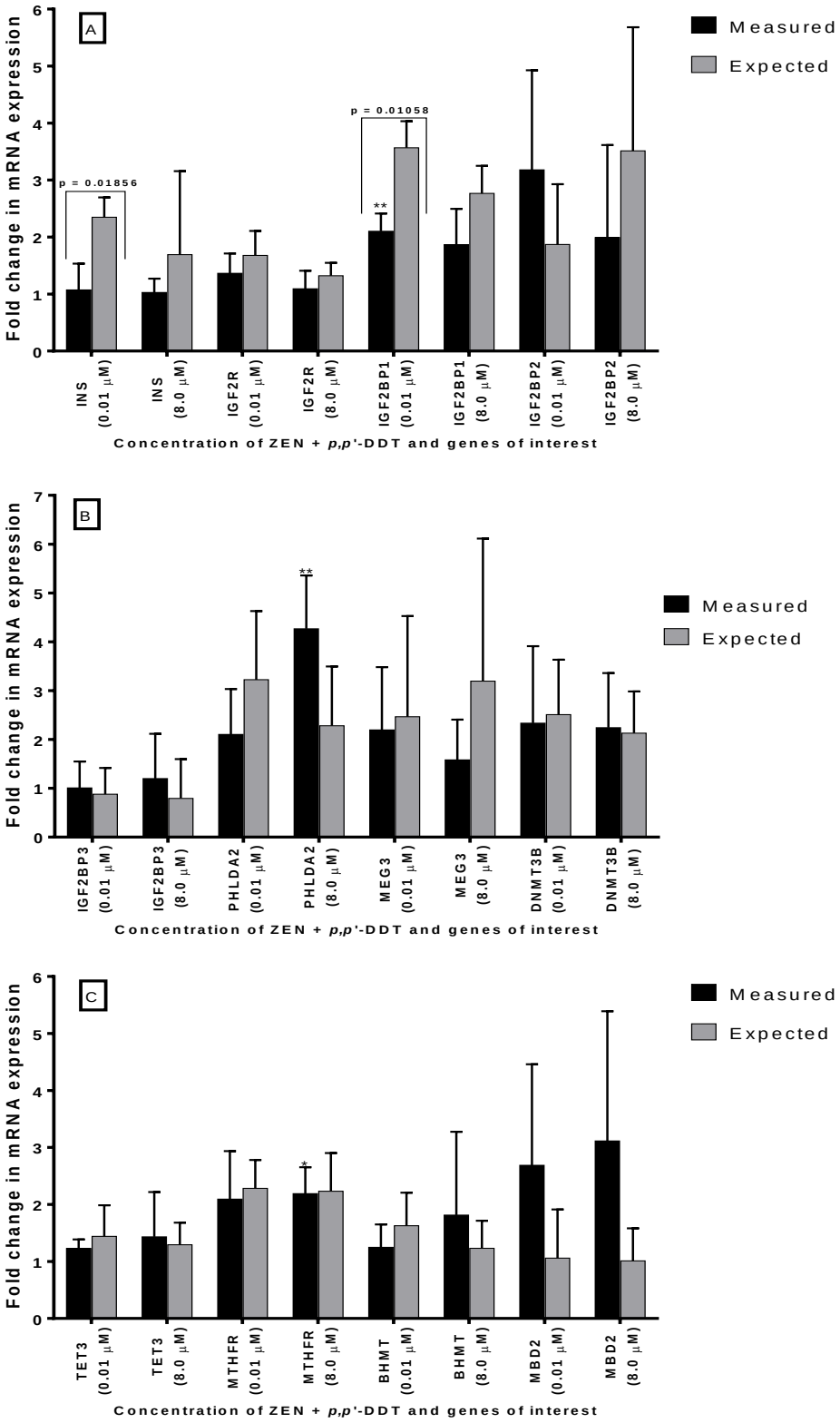

Fig. 5.17: Influence of binary mixtures of zearalenone (ZEN) and 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane (*p,p'*-DDT) on the mRNA expression of of genes for the IGF-axis, DNA methylation and genomic imprinting in BeWo cells using RT-qPCR.

The data represent the mean±SD of three biological replicates. Data were analysed using multiple t-test and corrected for multiple comparsion with Holm-Šídák test method. p≤ 0.05*, p ≤ 0.01**, p ≤ 0.001*** and p ≤ 0.0001**** represents significant fold change in mRNA expression and deviation from additve effects.

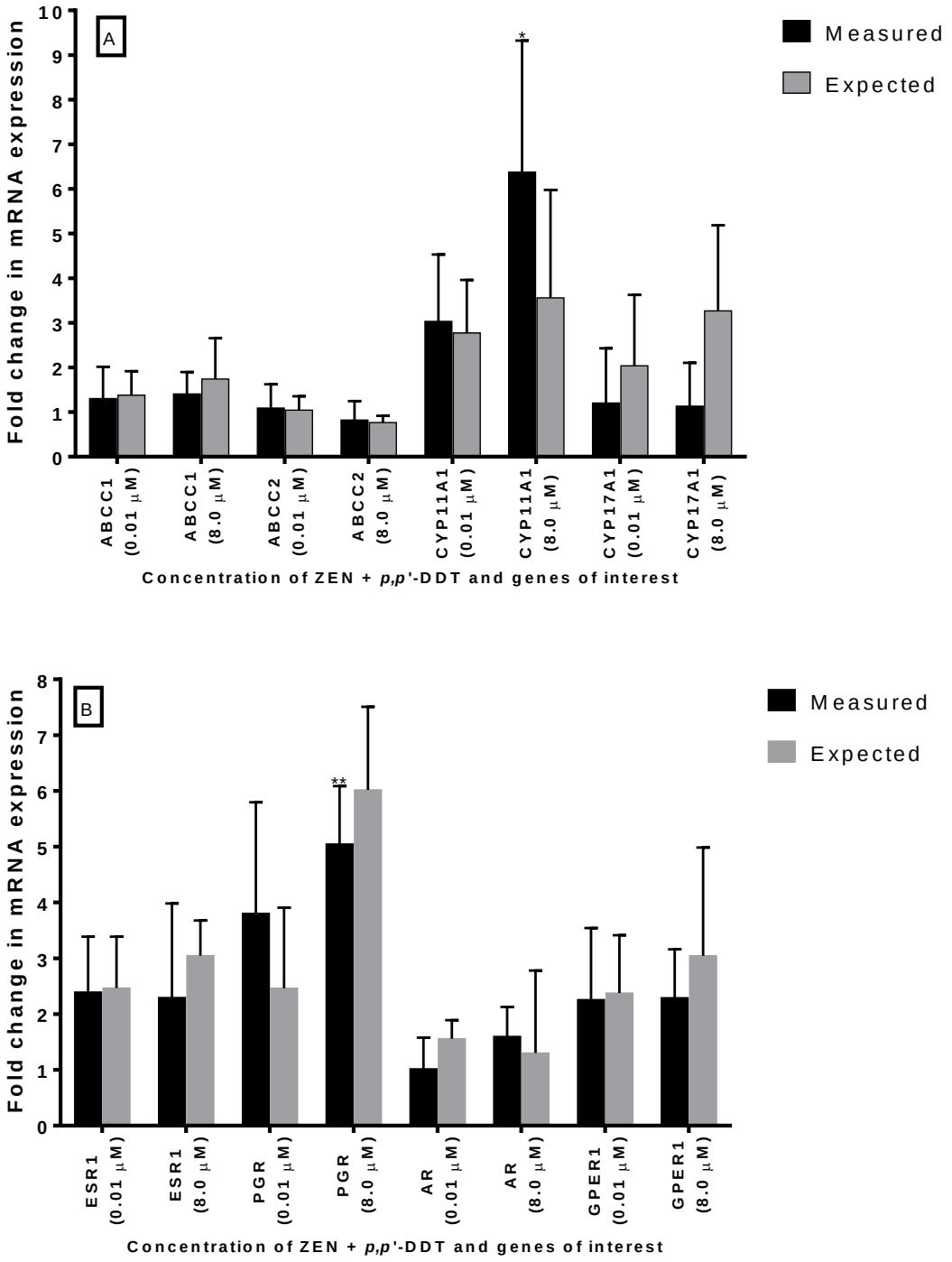

Fig. 5.18: Influence of binary mixtures of zearalenone (ZEN) and 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane (*p,p'*-DDT) on the mRNA expression of genes for plancental differentiation and angiogenesis in BeWo cells using RT-qPCR. The data represent the

mean±SD of three biological replicates. Data were analysed using multiple t-test and corrected for multiple comparsion with Holm-Šídák test method. $p \leq 0.05$*, $p \leq 0.01$**, $p \leq 0.001$*** and $p \leq 0.0001$**** represents significant fold change in mRNA expression and deviation from additve effects.

5.3 Discussion

Globally, multiple mycotoxin exposure biomarkers have been reported in several human epidemiological studies confirming that human beings are concurrently exposed to mixtures of mycotoxins (Solfrizzo et al., 2014). Similarly, worldwide surveys indicate that foods and feedstuff are continuously being contaminated by two or more mycotoxins (Rodrigues and Naehrer, 2012; Schatzmayer and Streit, 2013; Streit et al., 2013) and in some cases, with pesticides (Musaiger et al., 2008; Romero-González et al., 2011). Despite the fact that humans can be exposed to a range of mixtures of environmental and food contaminants, most toxicological studies have only taken into account of the effects of exposure to a single chemical. Studying the toxicological effects of chemical mixtures is of public health importance since chemicals in a mixture can interact and produce various effects, including synergistic, additive or antagonistic effects. Therefore, this study considered the cytotoxicity of individual and binary mixtures mycotoxins and/or pesticides in a placental cell line. The selected mycotoxins and persistent organochloride pesticides have been associated with reproductive and developmental toxicity in humans and animal models (ATSDR, 2002; Shuaib et al., 2010a; Frizzell et al., 2011, 2013; Malir et al., 2013; Cortinovis et al., 2013; EFSA, 2017; Yu et al., 2017).

5.3.1 Cytotoxicity of individual mycotoxins on BeWo human placental cells

In the experiment using single mycotoxins, OTA was the most cytotoxic followed by DON, ZEN, and β-ZOL, with AFB1 and α-ZOL showing slight toxicity at all the concentrations tested. It is well established that OTA is carcinogenic, hepatotoxic and a potent nephrotoxin, but report on its effect on reproduction are limited (Malir et al., 2013).

5.3.1.1 Cytotoxicity of OTA, DON and AFB_1 on BeWo cells

Among the tested mycotoxins (**Fig. 5.1**), OTA (IC_{50}: 10.21 ± 0.21 µM) was the most cytotoxic to BeWo cells followed by DON (IC_{50}: 13.39 ± 1.12 µM), β-ZOL (IC_{50}: 23.71 ± 0.05 µM), and ZEN (IC_{50}: 33.97 ± 2.45 µM). OTA also induced strong cytotoxicity in JEG-3 human placental cell line exposed at concentrations of 1 µM and above (Woo et al., 2013). The results of this study regarding OTA are similar to results in HepG2 cells (IC_{50}: 8.9 µM) and embryo porcine fibroblast (IC_{50}: 10.5 µM), but lower than that reported in Chinese hamster lung cell line (V79) (IC_{50}: 19) (Fusi et al., 2010; Behm et al., 2012; Wang et al., 2014). In a Chinese hamster ovary fibroblast cell line (AA8), OTA was also very cytotoxic with a reduction in cell viability occurring at 10 µM and above and a low IC_{50} value (IC_{50}: 17 µM), similar to the result reported here (Cosimi et al., 2009). In the same study (Cosimi et al., 2009), OTA inhibited the catalytic function of DNA topoisomerase II, and induced DNA damage and polyploidy in exposed AA8 cells. Also in agreement with the result of this study, OTA caused dose-dependent cytotoxicity in the V79 Chinese hamster cell line and human lymphocytes *in vitro* (Mosesso et al., 2008). It has been speculated that cell lines and primary cells derived from the kidney are more sensitive to the toxic effects induced by OTA compared to other cells (Schwerdt et al., 1999; Gennari et al., 2004; Horvath et al., 2002; Kamp et al., 2005). This has been attributed to the ability of OTA to induce oxidative damage and cell apoptosis, and disrupt mitosis, mitochondrial phosphorylation and protein synthesis in exposed cells (Malir et al., 2016). In contradiction to the result of this study, Caco-2 cells (Clarke et al., 2014) and MA-10 Leydig cells (**Chapter 4**) were less susceptible to OTA exposure, especially at lower concentrations. However, OTA was found to be cytotoxic to murine RAW 264.7 macrophage (RAW 264.7) and Madin-Darby Bovine Kidney (MDBK) cells at 24.8 µM reducing cell viability to 33% and 55%, respectively (Clarke et al., 2014). The strong toxicity exhibited by OTA in placental cells have been suggested to be due to OTA is a substrate of the influx transporter organic anion transporter 4 (OAT4) and ATP-binding cassette (ABC) transporters such as breast cancer resistance protein (BCRP, ABCG2) and multidrug resistance-related protein 2 (MRP2, ABCC2) which are involved in the movement of xenobiotics across the placental barrier (Cha et al., 2000; Berger et al, 2003; Schrickx et al., 2006; Woo et al., 2012).

In vivo, it has been postulated that OTA can cross the placental barrier and cause placental toxicity and *in utero* foetal exposure, resulting in interference of foetal development and possible adverse health outcomes (Braicu et al., 2010; Woo et al., 2012). In pregnant animals exposed to OTA, teratogenic effects and malformations were found in the embryo and foetus of mice (Hayes et al., 1974; Arora and Frölén, 1981; Arora et al., 1981), rabbits (Wangikar et al., 2004a), and rats (Wangikar et al., 2004b, 2004c). Increased embryo resorptions, post-implantation losses and low foetal weights have also been reported in animals exposed to OTA prenatally (Wangikar et al., 2004a; Wangikar et al., 2004b).

DON can be transferred through the placenta to the foetus in pregnant sows (Goyart *et al*., 2007; Dänicke *et al*., 2007) and it is likely that the human foetus will be exposed to DON during pregnancy. In an *in vitro* placental perfusion study using human placental BeWo cell line, Nielsen *et al*. (2011) showed that DON was transported across the membrane. In this current study, BeWo cell line was exposed to graded concentrations of DON to evaluate its effect on cell viability. DON was found to be strongly cytotoxic to BeWo cells in dose-dependent manner with an IC_{50} of 13.39 ± 1.12 µM). Strong cytotoxic effects have also been reported for DON in BeWo cells with an IC_{50} of 21 µM (Nielsen et al., 2011), Caco-2 cells with an IC_{50} of 1.4 - 13.0 µM (Alassane-Kpembi et al., 2013; Vejdovszky et al., 2016) and porcine intestinal cell line (IPEC-1) with IC_{50} of 16.54 µM (Alassane-Kpembi et al., 2015). Also in accordance with this study, DON was reported to be cytotoxic to renal proximal tubule epithelial cells, human lung fibroblast, Chinese hamster ovary K1 cells, human haematopoeitic progenitors, porcine jejunal epithelial (IPEC-J2) cell line, HepaRG and HepG2 liver cell lines, and RAW 264.7 cell line (Konigs et al., 2007; Ruiz et al., 2011a; Wan et al., 2013; Ficheux et al., 2012; Smith et al., 2017b; Zhou et al., 2017). In bovine granulosa cells, DON had no effect on proliferation (Albonico et al., 2016; Pizzo et al., 2015, 2016) whereas other studies on porcine granulosa cells shows contradictory results of the effects of DON, indicating either inhibitory activity (Ranzenigo et al., 2008; Cortinovis et al., 2014) or a stimulatory effect (Medvedova et al., 2011) which were dose and time dependent. In porcine oocytes exposed to DON *in vitro*, malformations of the meiotic spindle, inhibition of cumulus expansion and induction of cumulus cell death were observed (Malekinjad et al. 2007; Schoevers et al. 2010; Han et al. 2016). Similarly, DON

reduced oocyte quality and developmental competence of ovaries in mouse (Hou et al. 2014). The cytotoxic effects of DON on different cell lines has been attributed to its ability to inhibit DNA synthesis, induce ribotoxic stress, cellular cell-cycle arrest and apoptosis (Payros et al., 2016). Evidence indicate that the findings of this study may have clinical relevance. For instance, urinary concentrations of 436 ng/mL (1.471 µM) and 1,238 ng/mL (4.178 µM) have been reported for DON and its conjugates in pregnant women (Sarkanj *et al*., 2013; Wells *et al*., 2016).

AFB_1 has been detected in maternal sera and cord blood in different human epidemiologicl studies, and was associated with intrauterine growth restriction, still birth and growth faltering in early childhood (De Vires et al., 1989; Wild et al., 1991; Turner et al., 2007). In this study (**Fig. 5.1**), AFB_1 was not cytotoxic to BeWo cells, except at the highest concentration tested (64 µM). Similar non-cytotoxic effects of AFB_1 on MA-10 Leydig cell line (**Chapter 4**) and Caco-2 and RAW 264.7 cells (Clarke et al., 2014) have been described. However, AFB_1 was more cytotoxic to HepG2 cells than ZEN which could be as a result of the metabolic ability of HepG2 cells towards AFB_1 (Zhou et al., 2017). In addition, AFB_1 at 3 µM was cytotoxic to JEG-3 cells although the exposure time was 72 h (Storvik et al., 2011). Strong cytotoxicity of AFB_1 has also been observed in other studies using Caco-2 cells (Zhang et al., 2015), porcine Kidney (PK-15) cell line (Lei et al., 2013), and human umbilical vein endothelial cells (HUVEC), human lung fibroblasts and A2789 ovarian cancer cells (Braicu et al., 2010). The low toxicity of AFB_1 found in BeWo cell line could be attributed to its lack of metabolic capacity to transform AFB_1 to the toxic metabolites such as AFB_1-8-9-epoxide, aflatoxin Q1, aflatoxicol or AFB_1-dialdehyde which may have been caused by the suppression of cytotchrome P450 (CYP) 1A1 and aryl hydrocarbon receptor (AhR) activity in BeWo cells (Kensler et al., 2011). In JEG-3 placental cell line and human placenta explant cells, *p,p'*-DDT and its metabolites inhibited the activity of CYP1A1 and AhR, and protein expression after 48 h of exposure (Wójtowicz et al., 2011). It was also observed that co-treatment of *p,p'*-DDT and its metabolites with AhR agonist 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) alleviated the inhibitory action of *p,p'*-DDT and its metabolites on CYP1A1 and AhR activity in the JEG-3 placental cell line and human placenta explant cells (Wójtowicz et al., 2011). Arguably, it is possible that

AFB_1 exhibits similar inhibitory effects on CYP1A1 and AhR activity in BeWo cell line.

5.3.1.2 Effects of Zearalenone and its major metabolites on BeWo cell viability

ZEN and its major metabolites, α-ZOL and β-ZOL were not cytotoxic to BeWo cells at 16 µM after 48 h of exposure. Previous studies using different placental cell lines show that ZEN, α-ZOL and β-ZOL at ≤ 10 µM were not cytotoxic to BeWo cells (Prouillac et al., 2009, 2012) and JEG-3 cells (Huuskonen et al., 2015) after 48 hours of exposure. There were variations in the cell viability for ZEN, α-ZOL and β-ZOL with β-ZOL showing the highest cytotoxicity (IC_{50}: 23.71 ± 0.05 µM) followed by ZEN (IC_{50}: 33.97 ± 2.45 µM) whereas α-ZOL (IC50: > 64 µM) was the least cytotoxic to BeWo cells. In agreement with the result of this study, ZEN (IC_{50}: 20 µM) was found to be more cytotoxic to BeWo cells, followed by α-ZOL (IC_{50}: 50 µM) whereas β-ZOL (IC_{50}: 75 µM) was least toxic after 48 h of treatment (Prouillac et al., 2009, 2012). It is well established that α-ZOL has the strongest oestrogenic potency compared to ZEN, with β-ZOL having the lowest oestrogenic activity (EFSA, 2017b). This could explain the differences in the IC_{50} obtained in BeWo cells. In subsequent studies, the IC_{50} of ZEN was 39.88 µM to 41.28 µM in HepG2 cells (Wang et al., 2014; Li et al., 2014a), 20 µM in Caco-2 cells (Abid-Essefi et al., 2009) and 10 µM in HepaRG cells (Smith et al., 2017b) whereas it was higher in CHO-K1 cells (IC_{50}: 60.30 µM; Tatay et al., 2014a). Some studies also reported that ZEN was more cytotoxic compared to α-ZOL depending on cell type. For instance, ZEN was found to be more cytotoxic to HepG2 cells than α-ZOL with IC_{50} values of 20 µM and 80 µM, respectively (Abid-Essefi et al., 2009). In another study using V9 cells, Behm et al. (2013) also found ZEN with an IC_{50} of 26 µM was more efficient in reducing cell viability compared to α-ZOL with an IC_{50} of 50 µM. In disagreement with this study, α-ZOL was more cytotoxic than ZEN in CHO-K1 cells (Tatay et al., 2014a), Vero cell line (Othmen et al., 2008) and HepG2 cells (Wang et al., 2014). In agreement to the result of this study, β-ZOL strongly inhibited bovine granulosa cell proliferation whereas α-ZOL had no significant effect on the growth of bovine granulosa cells (Albonico et al., 2016; Pizzo et al., 2015, 2016) or bovine granulosa cells (Ranzenigo et al., 2008). However, Ayed et al. (2011) found that the α-ZOL was the more cytotoxic ZEN metabolite than β-ZOL after 24 h of exposure in HeLa and Vero cells. The variations in the cytotoxicity of ZEN and its major

metabolites reported in different studies can be as a result of the use of different cell types, media composition, exposure concentration and duration or the metabolic capacity of the cells used (Ruiz et al., 2011b; Tatay et al., 2014a). For instance, ZEN metabolites (α-ZOL and β-ZOL) were not detected after the ability of BeWo cell line to biotransform ZEN to its major metabolites was evaluated (Prouillac et al., 2009). In agreement, ZEN was not converted to α-ZOL and β-ZOL in CHO-K1 ovarian cells (Tatay et al., 2014a) and HepG2 cells (Tatay et al., 2014b), although other unidentified dehydroxylated and glycosylated metabolites were detected in cells treated with α-ZOL. On the other hand, ZEN was converted to α-ZOL and β-ZOL as well as α-zearalanol (α-ZAL) and β-zearalanol (β-ZAL), but not zearalanone (ZAN) in JEG-3 cell line (Huuskonen et al., 2015). In human Caco-2 cells, ZEN crossed the cell monolayer and was metabolised to α-ZOL, β-ZOL, ZEN-glucuronide and α-ZOL-glucuronide (Videmann et al., 2008).

5.3.2 Cytotoxic effects of individual persistent organochloride pesticides

The *p,p′*-DDT and its major metabolite *p,p′*-DDE are known to cause poor reproductive outcome. The major exposure route to *p,p′*-DDT and its major metabolites is through dietary intake and these leads to their accumulation in lipid phases of the tissues and cells such as blood, adipose tissue, ovarian fluid and the placenta (Rogan and Chen, 2006). In *in vivo* and *in vitro* systems, *p,p′*-DDT and its major metabolite *p,p′*-DDE are oestrogen receptor agonists and induce oestrogenic effects in exposed animals and humans. In addition, *p,p′*-DDE is a potent androgen receptor antagonist (Kelce et al., 1995). In animals, *p,p′*-DDT exposure during the gestational period caused foetal resorption, miscarriage, stillbirth, reduced anogenital distance and nipple retention in males (Kelce et al., 1995; Krieg et al., 2016). In humans, *in utero* exposure to *p,p′*-DDT and metabolites is associated with reduced anogenital distance, cryptorchidism and urogenital malformation in male neonates (Longnecker et al., 2002; Salazar-Garcia et al., 2004; Bornman et al., 2010). Some studies have shown that *p,p′*-DDT exposure is associated with an increase in time to conception, intrauterine growth restriction and spontaneous miscarriage in humans (Krieg et al., 2016).

In the present study, *p,p′*-DDT was more cytotoxic to BeWo cells than *p,p′*-DDE with IC_{50} values of 26.60 ± 1.97 µM and > 64.0 µM, respectively. In human endometrial Ishikawa cell line, *p,p′*-DDT metabolite *o,p′*-DDT (50 µM) reduced cell viability to approximately 38% after 48 hours treatment (Frigo et al., 2005). When the cytotoxic effect of *p,p′*-DDT in HepG2 and Caco-2 cell lines was determined using MTT and neutral red uptake assay, *p,p′*-DDT reduced cell viability in both cell lines at concentrations above 10 µM, with cell viability reaching 10 – 30% at 100 µM (Takakura et al., 2013). In a subsequent study, *p,p′*-DDT metabolite *o,p′*-DDT at 10 µM and 100 µM reduced the cell viability of human endometrial cells to 46 ± 10.4% and 19.1 ± 9.1 %, respectively and induced apoptosis and necrosis in exposed cells (Bredhult et al., 2007). Similarly, *p,p′*-DDT at concentrations ≥ 30 µM decreased cell viability, and induced oxidative stress and apoptosis in rat Sertoli cells exposed *in vitro* (Song et al., 2008, 2011; Shi et al., 2009). Different mechanisms through which *p,p′*-DDT and its metabolites cause cytotoxic effects have been postulated. In human embryonic kidney (HEK 293) and human endometrial Ishikawa cells, *p,p′*-DDT and its metabolites induced activator protein – 1 (AP-1) and tumour necrosis factor-alpha (TNF-α) gene expression through the mitogen-activated protein kinase (MAPK) signaling pathawy resulting to apoptosis and cell death (Frigo et al., 2002, 2005). In another studies, *p,p′*-DDT and its metabolites caused perturbation of the AhR/CYP1A1 pathway by inhibiting the expression of AhR and CYP1A1 proteins in a human placenta explant thereby disrupting the placental metabolic and detoxification activity (Wójtowicz et al., 2011).

5.3.3 Cytotoxic and interactive effects of binary mixtures of mycotoxins and persistent organochloride pesticides

Generally, the combinations of mycotoxins resulted in synergistic, additive or antagonistic interactive effects in BeWo cell line after 48 h of exposure. The result of the present study showed additive and antagonistic effects at low and high concentrations, respectively when BeWo cells were exposed to mixtures of OTA and AFB_1 (**Table 5.1**). OTA and AFB_1 mixtures were reported to exhibit additive interactive effects in Vero kidney cells (Golli-Bennour et al., 2010), HepG2 cell line (Corcuera et al., 2011), and Caco-2, MDBK and Raw 264.7 cells (Clarke et al., 2014; Clarke et al., 2015). The cytotoxic interactive effects of OTA with other mycotoxins

have also been reported. The combination of OTA with ZEN or α-ZOL produced antagonistic effects in HepG2 cell line whereas ZEN + α-ZOL showed either additive or synergistic effects depending on concentration (Wang et al., 2014c). In this study (**Table 5.1**), OTA + ZEN showed additive effects at low doses and antagonistic effects at high dose combinations whereas OTA + α-ZOL produced synergistic effects at low concentrations and antagonistic effects at high concentrations. It has been suggested that the antagonistic effects found after co-exposure to OTA and ZEN could possibly be due to the ability of both mycotoxins to induce oxidative stress and apoptosis at high concentrations (Li et al., 2014c). Furthermore, DON and ZEN mixtures exhibited additive interactive effects at 0.1µM to 8 µM, but showed antagonistic effects at concentrations above 16 µM (**Fig. 5.5B; Table 5.1**). Kouadio et al. (2007) reported additive effects of DON and ZEN mixtures after 72 h exposure on Caco-2 cells while antagonistic effects were found in HTC116 human colon cancer cells after exposure to high doses of binary mixtures of DON and ZEN (Bensassi et al., 2014). In disagreement with this study, binary combination of DON and ZEN elicited synergistic interaction in HepaRG human liver cell line (Smith et al., 2017b) and IPEC-J2 porcine intestinal epithelial cells (Wan et al., 2013), although these authors used non-constant ratio of DON and ZEN combinations in their assay. Mixtures of DON with AFB_1 or ZEN produced additive or synergistic effects while combination of ZEN with AFB_1 produced antagonistic effect after 48 h exposure in HepG2 and RAW 264.7 cell lines (Zhou et al., 2017), similar to the result of this study. Similar synergistic effects were observed in porcine kidney 15 cell line after exposure to mixtures of DON and AFB_1 (Lei et al., 2013). The synergistic effects observed after exposure of HepaRG cells to DON and ZEN combinations was attributed to the induction of cytochrome P450 (*CYP) 3A4* and *CYP4F3B* enzyme mRNA expression which may have mediated the formation of more active metabolites (Smith et al., 2017b).

The binary combinations of ZEN and its metabolites α-ZOL and β-ZOL produced synergistic effects at concentrations ≤ 16 µM whereas at concentrations > 16 µM, antagonistic effects were observed in this study (**Fig. 5.6; Table 5.1**). In HepG2 cells, ZEN + α-ZOL, ZEN + β-ZOL and α-ZOL + β-ZOL exhibited synergistic interactive effects at low concentrations, and antagonistic and/or additive effects at high concentrations (Tatay et al., 2014b). On the contrary, binary combinations of ZEN, α-

ZOL and β-ZOL produced additive effects at both low and higher concentrations in CHO-K1 ovarian cell line after 48 h (Tatay et al., 2014a). In bovine granulosa cells, α-ZOL + β-ZOL, α-ZOL + DON and β-ZOL + DON produced additive interactive effects after 48 h (Pizzo et al., 2016). Taken together, the interactive effects exhibited by mycotoxin mixtures are dependent on the cell type, class of mycotoxins in the mixtures, the concentration ratio used for each mycotoxin in the mixture, the concentration range of the single mycotoxins, the method used to determine the interactive effects, media composition, exposure duration and the metabolic capacity of the target cell (Ruiz et al., 2011b; Tatay et al., 2014a).

Since there is co-contamination of mycotoxins with pesticides in food ingredients, especially in middle and low income countries, this study also evaluated the interactive effects of such co-exposure. Binary combination of *p,p'*-DDT with DON, OTA, ZEN, α-ZOL or β-ZOL produced either synergistic or additive effects in BeWo cell line at low concentrations, depending on type of mycotoxin and dose combinations. Similarly, mixtures of *p,p'*-DDE with DON, OTA, ZEN, α-ZOL or β-ZOL produced either synergistic and/or additive effects (**Fig. 5.3; Table 5.1**). In chapter 4, it was demonstrated that the cytotoxic effects of DON and OTA were enhanced by co-exposure with either *p,p'*-DDT or *p,p'*-DDE in MA-10 Leydig cells.

5.3.4 Effects of mycotoxins and persistent organochloride pesticides, either in single or binary mixtures, on placental endocrine function and gene expression

Despite the overwhelming evidence that co-exposure to mycotoxins and persistent organochloride pesticides are common in human beings, data on effects of these pollutants on placental and foetal health are scarce. Considering that *in utero* exposure to these contaminants has been reported in various studies (Cortinovis et al., 2013; Rogan and Chen, 2005), in the present study, BeWo placental cell line was used to determine their potential negative impacts on pregnancy and foetal health. The result of this study shows that ZEN and its metabolites had stimulatory effects on E2 release in BeWo cells, but had no effect on P4 level whereas *p,p'*-DDT had inhibitory effect on both E2 and P4 concentration. Most importantly, all the tested individual compounds significantly reduced β-hCG levels in cell media supernatant compared to DMSO

control. Binary mixtures of mycotoxins and *p,p'*-DDT pesticide differentially affect hormone production which were dependent on concentration and type of mixtures. In the gene expression assay, ZEN and *p,p'*-DDT, alone or in combination, significantly upregulated the expression of genes of the IGF-axis (*IGF2BP1* and *IGF2BP2*), genomic imprinting (*PHLDA2* and *MEG3*) and DNA methylation (*DNMT3B, MTHFR* and *MDB2*). Taken together, it can be deduced that mycotoxins and *p,p'*-DDT pesticide affect placental endocrine and metabolic function through different mechanisms.

5.3.4.1 Effects of ZEN and its major metabolites on placental hormone production

In the current study, ZEN and metabolites significantly increased E2 level in BeWo placental cells (**F.g. 5.8**; $p \leq 0.05$). In addition, ZEN and α-ZOL had no significant effect on P4 secretion, but β-ZOL significantly increased P4 level at 16 µM (**F.g. 5.7**; $p \leq 0.05$). It is well known that ZEN and metabolites bind to oestrogen receptors [ESR1 (ER-α) and ESR2 (ER-β)] disrupting the binding of E2 and also interfere with genes encoding for cytochrome P450 (CYP) and hydroxysteroid dehydrogenase (HSD) enzymes involved in steroidogenesis (Minervini and Dell'Aquila 2008). For instance, ZEN was reported to interfere with the expression of *3β-HSD-1* and *CYP11A1*, and also caused a decline in P4 and E2 levels in porcine primary granulosa cells cultured *in vitro* (Tiemann et al., 2003). On the contrary, ZEN did not affect *3β-HSD-1* and *CYP11A1* mRNA expression in granulosa cells from gilts fed with contaminated diet (Alm et al., 2006). Both α-ZOL and β-ZOL reduced both FSH and forskolin induced P4 release in procine granulosa cells after exposure to 15 µM and 30 µM of α-ZOL and β-ZOL *in vitro* and this coincided with a reduction of mRNA expression of *CYP11A1* and *3β-HSD* in the exposed cells (Tiemann et al., 2003). In a subsequent study, α-ZOL exhibited biphasic effects on IGF1-enhanced E2 production, significantly modulating oestradiol release at lower levels (0.094 - 0.936 µM) and decreasing its secretion at higher doses (9.36 µM) (Ranzenigo et al., 2008). In this same study (Ranzenigo et al., 2008), α-ZOL increased FSH-induced P4 production at lower concentrations (0.094 - 0.936 µM) and significantly increased IGF1-induced P4 production at higher concentrations. It has also been shown that α-ZOL did not affect E2 production, but increased P4 production in bovine granulosa cells (Cortinovis et al., 2014). The differences exhibited by ZEN and its metabolites on P4 release could be attributed to the differences in their oestrogenic potency as ZEN and α-ZOL are more oestrogenic compared to β-ZOL. Furthermore, the

β-ZOL-induced P4 secretion in BeWo placental cells could be caused by increased activity of 3β-HSD and CYP11A1 enzymes, and/or increased expression of placental StAR protein. However, this requires further studies. In an H295R adrenocarcinoma cell line, ZEN and its metabolites significantly increased both E2 and P4 levels in cell media (Frizzell et al., 2011). However, at 100 μM of ZEN, α-ZOL and β-ZOL, the concentrations of E2 was significantly reduced whereas P4 level remained unchanged. In a different study, ZEN also increased E2 level in H295R cells (Kolle et al., 2012) suggesting that it might be increasing *CYP19A1* mRNA level. Kolle et al. (2012) also observed that ZEN had biphasic effect on E2 production in H295R cells with increased production occuring at lower doses (0.03 μM - 10 μM) whereas E2 level decreased at 30 μM. This biphasic effect on E2 production was not observed in BeWo cells used in this present study. This could be as a result of either differences in cell line, the media composition or lower concentration of ZEN compared to that used by Kolle et al. (2012). Also similar to the result of this study, β-ZOL stimulated E2 and P4 production in bovine granulosa cells at higher concentration (31 μM) in the absence of IGF-1 *in vitro* and this increase in E2 level was supported by the increase of *CYP19A1* mRNA level (Pizzo et al., 2015, 2016). The CYP19A1 enzyme is the main enzyme involved in the conversion of testosterone to E2, indicating increased activity of this enzyme. In chapter 4 of this thesis, it was demonstrated that ZEN and its metabolites increased P4 levels in MA-10 murine Leydig cell line. This further highlights the advantage of using gonadal cell lines in assessing reproductive and developmental toxicity.

As β-hCG is considered as a biomarker of placental health and endocrine function, this study also determined the impact of ZEN and its metabolites on β-hCG secretion in BeWo placental cells. It was demonstrated that ZEN, α-ZOL and β-ZOL at all the doses tested (0.1 - 16 μM) significantly ($p \leq 0.001$) decreased β-hCG level in BeWo placental cell media supernatant. On the contrary, ZEN at 10 μM increased β-hCG production whereas α-ZOL and β-ZOL (10 μM, respectively) had no significant effect on β-hCG production in BeWo cells (Prouillac et al., 2012). However, a significant induction of *hCG* mRNA expression was observed after ZEN (10 μM) exposure while β-ZOL (10 μM) significantly inhibited *hCG* mRNA expression and no effect was observed with α-ZOL (10 μM) treatment (Prouillac et al., 2012). The differences observed could be as a result of the use of serum free media, number of cells (100,000 cells/well) and cell passage number (194 - 205) by Prouillac et al. (2012) compared to media containing 5%

charcoal-stripped foetal bovine serum, cell number (50,000 cells/well) and a cell passage of 12 - 25 as used in this study. Cell culture medium and supplementation has been shown to affect hCG production in human placental cells (Lanoix and Vaillancourt, 2010).

5.3.4.2 Effects of DON on placental hormone production

In this study (**Fig. 5.7 & Fig. 5.8),** DON exposure at 8 - 16 µM significantly reduced E2 and P4 levels in placental cell media after 48 h treatment ($p \leq 0.01$). DON is known to cause oxidative damage, inhibit DNA, RNA and protein synthesis, impair membrane integrity, induce apoptosis and upregulate the expression of genes involved in inflammation (Pestka, 2010a,b). However, there is little literature reporting its adverse effects on reproductive function, especially in animal models. For instance, porcine granulosa cells cultured with DON (1000 ng/mL; 3.3 µM) inhibited P4 production, follicle stimulating hormone (FSH) plus Insulin Growth factor 1 (IGF1)-induced *CYP19A1* and *CYP11A1* mRNA expression (Ranzenigo et al. 2008). On the contrary, Medvedova et al. (2011) reported an increased production of P4 in ovarian granulosa cells treated with 1000 ng/mL (3.3 µM) of DON. Consistent with these results, in a subsequent *in vitro* study using the H295R adrenocarcinoma cell line it was shown that DON (100 ng/mL) up-regulated the expression of steroidogenic genes, including *CYP1A1, CYP21, CYP17, HSD3B2, CYP11B1 and CYP11B2*, and down-regulated the expression *of CYP19*. Increased levels of DON (compared to T-2 and HT-2 toxins) also reduced cell viability, E2, testosterone and cortisol levels whereas P4 was significantly increased in cells treated with 1000 ng/mL (3.3 µM) of DON (Ndossi et al., 2012). The difference in the actions of 1000 ng/mL of DON on P4 production could possibly be as a result of the stimulation of cells with FSH and IGF1 prior to treatment with DON (Ranzenigo et al. 2008) compared to non-stimulation of cells before treatment with DON (Medvedova et al. 2011; Ndossi et al. 2012) or due to species differences. Recently, Cortinovis et al. (2014) observed that DON inhibited P4 and E2 production by 74% and 67%, respectively after co-incubation with either FSH or IGF1 in porcine granulosa cells. In a more recent study, DON was found to inhibit E2 and P4 secretion in bovine small -and large follicle granulosa cells after in vitro co-treatment with IGF1 (Pizzo et al., 2015, 2016), but increased E2 production in the absence of IGF1 (Pizzo et al., 2016). However, there was no effect on *CYP11A1* and *CYP19A1* mRNA level when

small granulosa cells were exposed to DON together with IGF1 (Pizzo et al., 2016). Also in agreement with the result of this study, exposure of bovine granuolsa cells with DON (0.3 µM) strongly inhibited E2 and P4 level, and reduced the mRNA expression levels of *CYP19A1* but did not alter StAR and *CYP11A1* levels (Guerrero et al., 2015). Taken together, it can be deduced that some of the mechanisms by which DON negatively impact reproduction and development is through the perturbation of hormone production and induction of apoptosis. The differences observed in various studies could be explained by the differences in media composition, cell type, treatment duration or the use of IGF1 and FSH which may produce protective effects on cells.

This study also evaluated the effect of DON on β-hCG level in BeWo cells after 48 h treatment. DON (0.1 - 16 µM) significantly ($p \leq 0.0001$) decreased level of β-hCG in BeWo cell media (**Fig. 5.9E**). Similar concentrations of DON used in this study also decreased β-hCG in BeWo cells after 24 h exposure (Nielson et al., 2011). The results of this study further support the maternal and embryo toxicity that has been reported in several studies in animals and the concentrations of DON used are of human relevance as such concentrations have been reported in the urine of pregnant women from UK (Hepworth et al., 2011; Wells et al., 2016), Croatia (Sarkanj et al., 2013), and Belgium (Heyndrickx et al., 2015).

5.3.4.3 Effects of OTA on placental hormone production

In this study, OTA decreased E2 level in BeWo cell line only at 8 µM and 16 µM (Fig. 5.8), while it had no significant effect at 1 µM but strongly inhibited P4 secretion at 16 µM (**Fig. 5.7F**). Ochratoxin A (OTA) is the most toxic of all the ochratoxins and studies indicate that OTA is nephrotoxic (associated with Balkan Endemic Nephropathy), hepatotoxic, neurotoxic, immunotoxic, genotoxic and carcinogenic, and have been classified as a possible human carcinogen (group 2B; IARC, 2002). However, less is known about the impact of OTA on reproductive health and also, the mechanisms involved in the adverse reproductive health outcome is largely unclear. In another *in vitro* model, OTA (1000 ng/ml) impaired steroidogenesis through the modulation of E2 production, but no effect on P4 level in adrenocarcinoma cell line (H295R cells) compared to the control (Frizzell et al. 2013). Also different from the report of this

study, OTA was reported to cause an increase in P4 production and upregulation of 3β-HSD activity in human choriocarcinoma cell line (JEG-3 cells) by 281–378% at 72 and 96 h (Woo et al. 2013). JEG-3 cell line has been shown to produce more P4 compared to BeWo cells (Drwal et al., 2017) and this could be the reason for the different response observed in both cell lines. Although it remains unclear how interference of steroidogenesis by these mycotoxins *in vitro* can be extrapolated to human reproduction, these findings are of clinical relevance since knowledge gained through *in vitro* models can be applied to predict toxicity in intact animals (Minervini and Dell'Aquila 2008). This current study is the first to report the effect of OTA on the placental secretion of β-hCG either *in vivo* or *in vitro*. OTA significantly ($p \leq 0.0001$) reduced β-hCG level in BeWo placental cell line at all the concentrations tested (0.1 - 16 µM; **Fig. 5.9F**) and this could pose a great risk to pregnancy.

5.3.4.4 Effects of *p,p'*-DDT on placental hormone production

This study also evaluated the effects of *p,p'*-DDT on the production of P4, E2 and β-hCG in BeWo cells. In this present study (**Fig. 5.7A**), it was observed that *p,p'*-DDT significantly reduced P4 secretion in BeWo cells at 8 µM and 16 µM ($p \leq 0.001$), but had no significant inhibitory effect on E2 production (**Fig. 5.8A**). In JEG-3 choriocarcinoma cell line, *p,p'*-DDT at 3 µM significantly inhibited P4 release after 24 h exposure, but exhibited stimulatory effect on P4 secretion after 72 h treatment (Wojtowicz et al., 2007a), indicating time specific effects. Similar data were reported by Wojtowicz et al. (2007c) who found that after 24 h of exposure, *p,p'*-DDT concentrations between 1 µM and 16 µM strongly inhibited both P4 and E2 levels in prepubertal porcine ovarian follicles. In female rabbits, *p,p'*-DDT exposure caused a significant reduction in P4 production and this had adverse effect on their ovulation rate (Lindeau et al., 1994). In steroidogenesis, the rate of P4 formation is dependent on the mobilisation of cholesterol into the inner mitochondrial membrane by StAR and the conversion of cholesterol to pregnenolone by CY11A1. Therefore, the reduction of P4 level by *p,p'*-DDT treatment could be as a result of low level of substrates required for P4 biosynthesis or due to the inhibition of CYP11A1 activity. In addition, P4 is the major substrate for testosterone synthesis which is later metabolised to E2 by CYP19A1. It is possible that decreased P4 level in BeWo cells resulted in low testosterone production or that CYP19A1 activity was inhibited by *p,p'*-DDT, and

consequently, low E2 level as observed. The earlier is supported by the work of Wojtowicz et al. (2007c) who reported that the addition of external testosterone to the cell culture media of ovarian follicles treated with *p,p′*-DDT reversed the inhibitory effect previously reported for E2 level confirming that lack of T caused the reduction in E2 secretion in the exposed cells. In human full term placental explants, *p,p′*-DDT significantly inhibited CYP19A1 activity resulting in the inhibition of the conversion of dehydroepiandrosterone to E2 (Wojtowicz et al., 2007b). Inhibition of CYP19A1 activity after *p,p′*-DDT treatment has also been described in H295R human adrenocortical carcinoma cells (Sanderson et al., 2002).

The placenta is the major source of β-hCG in the early and late stages of pregnancy, and its presence stimulates placental P4 production. The result of this present study (**Fig. 5.9A**) showed that *p,p′*-DDT (0.1 – 8 µM) also inhibited β-hCG level in BeWo placental cell line after 48 h exposure. In JEG-3 placental cell line, *p,p′*-DDT treatment at about 3 µM inhibited β-hCG after 24 h, but stimulated β-hCG after 72 h exposure (Wojtowicz et al., 2007a). It has also been shown that 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) decreased β-hCG level in cell media supernatant and inhibited P4 production in JEG-3 cells (Augustowska et al., 2007). The reduction of P4 in BeWo cells treated with *p,p′*-DDT could be explained by the decline of β-hCG release which indirectly stimulates P4 secretion in placental cells.

5.3.4.5 Effects of binary combinations of mycotoxins and *p,p′*-DDT pesticide on BeWo cell hormone production

To date, no studies have been carried out to assess the in vitro effects and the mechanism of action of mycotoxin and pesticide mixtures on placental endocrine function. The present study also evaluated the effects of equimolar concentrations (0.1, 1 and 8 µM) of *p,p′*-DDT and DON in combination with ZEN and its metabolites on BeWo cell hormone production (**Fig. 5.10 - Fig. 5.12**). These selected combinations had cytotoxic effects ≤ 20% on BeWo cell line (**Fig. 5.1**). Note worthy, binary combinations of *p,p′*-DDT or DON with ZEN synergistically reduced E2 production in dose-dependent fashion, indicating that these toxins had antagonistic effects on ZEN-induced E2 secretion. Similarly, combinations of *p,p′*-DDT or DON with either α-ZOL or β-

ZOL reduced E2 level in BeWo cell media supernatant at 0.1 µM and 1 µM. Although the E2 level improved at 8 µM, this did not reach the concentrations observed in α-ZOL or β-ZOL, alone at this concentration (8 µM) suggesting interaction between *p,p'*-DDT and DON with these ZEN metabolites. Also worthy of note is that combination of *p,p'*-DDT or DON with ZEN, α-ZOL or β-ZOL decreased (0.4 - 0.7 fold) P4 production dose-dependently. The inhibitory effects observed on P4 secretion when p,p-DDT or DON were combined with ZEN and its metabolites suggest that *p,p'*-DDT or DON acted additively with ZEN and its metabolites to impair P4 secretion in BeWo cells. Exposure of bovine small follicle granulosa cells to binary combinations of α-ZOL with DON or β-ZOL in the presence of IGF1 inhibited E2 secretion higher than the effects obtained in single treatment of each of the toxins, but had no significant effect on P4 production (Pizzo et al., 2016). In bovine large granulosa cells, co-exposure of DON and α-ZOL inhibited FSH-induced progesterone secretion *in vitro*, but induced E2 level although this was not significantly compared to either DON or α-ZOL alone (Pizzo et al., 2015). However, in the presence of both IGF1 and FSH, combination of the two mycotoxins had no effect on E2 and P4 release compared to controls. This current study also demonstrated that equimolar combinations of α-ZOL with ZEN or β-ZOL had no effect on E2 release at 0.1 µM and 1 µM compared to DMSO control, but significantly increased E2 concentration at 8 µM. On the contrary, these combinations had little or no effect on the ability of BeWo cells to secrete P4 in media. In accordance to the result of this study, combination of α-ZOL and β-ZOL also inhibited E2 production compared to control, but had no effect on P4 level in bovine small granulosa cells *in vitro* (Pizzo et al., 2016). As regards β-hCG production in BeWo cells, combination of *p,p'*-DDT or DON with ZEN, α-ZOL or β-ZOL induced the secretion (2 fold), especially at 0.1 µM equimolar concentrations whereas *p,p'*-DDT/ β-ZOL, DON/ZEN, DON/α-ZOL, and DON/β-ZOL negatively affected β-hCG production, particularly at 8 µM, but no effect was observed for α-ZOL/β-ZOL in all the concentrations tested.

5.3.4.6 Effects of ZEN and *p,p'*-DDT in single or mixtures on BeWo cell gene expression

Several experiments examining placentas from intra-uterine growth restricted pregnancies and normal placentas have shown a pattern of differential expression of imprinted genes involved in placental growth and foetal development (Moore et al.,

2015). The maternally expressed imprinted gene pleckstrin homology like domain, family A, member 2 (*PHLDA2*) has been suggested to play a critical role in regulating foetal growth and birth weight of infants, and alteration in the expression of this imprinted gene may cause intra-uterine growth restriction (Lim et al., 2012). For instance, the evaluation of the gene expression profile of umbilical cord blood samples obtained from Chinese infants demonstrated that elevated expression *PHLDA2* and lower expression of paternally expressed gene 10 (*PEG10*) mRNA level was correlated with low birth weight (Lim et al., 2012). In a systematic evaluation of 74 putatively imprinted genes in the placenta from ten normal and seven intra-uterine growth restricted (IUGR) pregnancies from USA women using RT-qPCR, it was observed that *PHLDA2*, *PEG10* and *MEST* expression were upregulated whereas *IGF2* and *PLAGL1* were downregulated in IUGR placentas (Diplas et al., 2009). The mRNA expression of imprinted genes have also been suggested to be associated with spontaneous abortion and foetal deaths. In a study comprising 54 samples (ovular fragments, feotal slice of skin from hill or thigh and placenta) from 38 women with spontaneous miscarriage or foetal death, it was demonstrated that elevated *PHLDA2* mRNA level was associated with spontaneous miscarriage or foetal death occurring in both first and second trimester (Dória et al., 2010). Other studies have shown similar findings that elevated expression of *PHLDA2* mRNA is found in placentas from low birth weight (Apostolidou et al., 2007) and IUGR (McMinn et al., 2006) pregnancies. The present study (**Fig. 5.14**) demonstrated that ZEN and *p,p'*-DDT in mixture significantly elevated the mRNA expression *PHLDA2* in BeWo placental cells suggesting that exposure to these toxins could have adverse effects on embryonic development, placental formation, foetal and postnatal growth.

In these studies (**Fig. 5.14**), ZEN and *p,p'*-DDT, in single or in mixtures affected the IGF-axis by elevating the mRNA expression of *INS*, *IGF2BP1* and *IGF2BP2*. In the preliminary experiments in this study, the mRNA expression of *IGF1*, *IGF1R* and other genes of the IGF-axis were examined, but *IGF1* and *IGF1R* fold change were not significantly different from the control. Therefore, these genes were not taken further in the study. The IGF-axis comprises key regulatory endocrine factors involved in pre- and postnatal growth and development and include insulin (*INS*), *IGF1*, *IGF2* and their associated receptors (*IR*, *IGF1R* and *IGF2R*) , and six binding proteins (*IGFBP1- 6*)

(Moore et al., 2015). The dysregulation of this pathway results to poor placental growth, embryonic and foetal development, and prenatal growth (Baker et al., 1993). It has been postulated that AFB_1 cause growth restriction by the alteration of the insulin-like growth factor (IGF) axis thereby affecting growth and development (Castelino et al., 2015). Reduced IGF1 mRNA and protein levels were observed in Kenyan school children exposed to AFB_1 *in utero* and in human hepatocyte cell line 16 (HHL-16) exposed to AFB_1 *in vitro* (Castelino et al., 2015). In agreement with the result of the present study, increased levels of plasma IGFBP2 and IGFBP4 were found in guinea pig foetuses with growth restriction while IGFBP3 levels were significantly reduced compared to controls, but no changes in *IGF1* and *IGF2* mRNA expression in growth restricted guinea pigs (Carter *et al.* 2005). In the same study, the *IGFBP2* mRNA expression also increased in the liver of IUGR foetuses (Carter *et al.* 2005). In a subsequent study, exposure of sheep foetuses to high concentration of testosterone *in utero* resulted in elevated serum IGFBP1 and IGFBP2 levels and this was well correlated with growth restriction in the foetuses (Manikkam *et al.* 2004), indicating testosterone may affect foetal growth by altering IGF pathway. Elevated expression of *IGFBP1* have been reported in another study on growth restricted placentas (Okamoto *et al.* 2006). It is well established that IGFBPs inhibit the biological function of IGFs by competing with the IGF receptors for the peptide. Therefore, impaired embryo and foetal development as generally observed in intrauterine growth restricted (IUGR) pregnancies could possibly be as a result of decreased availability of IGFs and increased expression of IGFBPs (Carter *et al.* 2005). Interestingly, exposure to other mycotoxins has been linked to disruption of the IGF growth factor pathway. For example, Amuzie and Pestka (2010) demonstrated that growth retardation observed in mice exposed to DON could be as a result of its ability to inhibit plasma IGF1 and IGF-ALS (insulin Like growth factor binding protein acid labile subunit) levels in mice, while it has been speculated that OTA causes reduction in *IGFBP1* and *IGF2* mRNA expression levels in the HepG2 human hepatocarcinoma cell line (Hundhausen *et al.* 2009).

ZEN, *p,p'*-DDT and *p,p'*-DDT/ZEN upregulated *LHCGR* expression level in BeWo cells (**Fig. 5.15**). In addition, *p,p'*-DDT elevated *LEP*, *DYSF* and *ERVW-1* while co-treatment of BeWo cells with DDT and ZEN downregulated *LEP* level, but upregulated mRNA expression of *ERVW-1*, *VEGF*, *MMP2* and ZFP57. Increased expression of

LEP, *VEGF*-R), *HCG*, and hypoxia-inducible factor 2α (*HIF-2α*) was observed in growth restricted placentas suggesting that hypoxia may have a role to play in placental growth (Mccarthy *et al.2007).* In a previous study, ZEN (10 µM) caused significant induction of *ERVW-1*, *ERVW-2* and *hCG* mRNA expression in BeWo cells after 48 h treatment (Prouillac et al. 2012), similar to the result of this study.

In this study, the level of DNA methylation were not assessed, however, the mRNA expression of some genes involved in DNA methylation were evaluated. Proper DNA methylation pattern is of critical importance in embryonic development, placental formation, fetal and postnatal growth. DNA methyltransferases (DNMT3A and DNMT3B) are responsible for the de novo methylation during early embryonic development (Reik et al., 2001). The present study demonstrated that *p,p'*-DDT exposure significantly elevated the mRNA expression of *MTHFR* in BeWo cells, and reduced *MBD2* mRNA expression, but these were not significantly different from the control. Interestingly, co-treatment with ZEN and *p,p'*-DDT strongly elevated the mRNA expression of *MTHFR*. This shows that exposure influences DNA methylation level in BeWo placental cells. It has been reported that DON treatment increased the DNA methylation level and the relative expression of DNMTs mRNA in porcine oocytes (Han et al., 2016). DON exposure at 3 µM reduced the maturation rate of porcine oocytes by affecting their epigenetic modifications through increase in global 5-methylcytosine (5mC) level and elevating the DNMT3a mRNA expression level (Han et al., 2016). It has been reported that ZEN exposure reduced the developmental competence of maturated mouse oocytes by significantly reducing 5mC level, but had no effect on the mRNA expression of DNMTs (Zhou et al., 2016). It was suggested that ZEN affected DNA methylation at protein level instead of the DNMTs mRNA transcripts (Zhou et al., 2016).

This study also examined the mRNA expression of the genes involved in steroidogenesis after exposing BeWo cells to ZEN, *p,p'*-DDT and *p,p'*-DDT/ZEN to understand the mechanisms involved in the effects these toxins and their combination on P4 and E2 production. *p,p'*-DDT and ZEN increased the expression of *CYP11A1* mRNA level. However, *p,p'*-DDT had no effect on P4 level at low dose (0.1µM), but

inhibited P4 level at 8 µM. It was expected that the increased *CYP11A1* mRNA expression could have caused an increase in P4 release as it is the rate-limiting enzyme involved in the conversion of cholesterol to pregnenolone. However, the reduction of P4 secretion at 8 µM of *p,p′*-DDT exposure could be either as a result of inhibition of the enzyme 3β-HSD which converts pregnenolone to P4 or the increased activity of CYP17A1 which converts P4 to 17α-hydroxyprogesterone. The later could be the reason as *CYP17A1* mRNA expression was also elevated by 8 µM of *p,p′*-DDT. The increased E2 level found in BeWo cells exposed to ZEN at 8 µM is supported by the increased mRNA expression of *CYP17A1* providing substrate for E2 production or an increased activity of *CYP19A1* which is responsible for the conversion of T to E2. In porcine granulosa cells, ZEN interfered with the level of *3β-HSD1* and *CYP11A1* (Tiemann et al., 2003). The binary of *p,p′*-DDT and ZEN also increased *CYP11A1* level, but had no significant effect on *CYP17A1* expression. Therefore, the reduction of both P4 and E2 in BeWo cells exposed to *p,p′*-DDT/ZEN, especially at 8 µM could be as a result of lack of substrates for P4 and E2 production or inhibition of the metabolic enzymes involved in the synthesis of both hormones. *p,p′*-DDT and ZEN mixture also acted synergistically to increase the expression level of *VEGF* and *MMP2* (**Fig. 5.15**) but acted antagonistically on *IGF2BP1* mRNA expression (**Fig.5.17A**).

5.5 Conclusion

This *in vitro* study has shown that BeWo cells are particularly susceptible to the cytotoxic effects induced by OTA, DON and *p,p′*-DDT. In addition, the cytotoxic effects of single mycotoxins were magnified by co-treatment with either *p,p′*-DDT or *p,p′*-DDE. This study further provides evidence that single or mixtures of mycotoxins and *p,p′*-DDT pesticide can affect placental hormone production, and is the first to evaluate the interaction of ZEN and *p,p′*-DDT on the expression of placental imprinted genes. Influencing placental endocrine function, DNA methylation and genomic imprinting could be other mechanisms through which mycotoxins and persistent organochloride pesticides, alone or in combination, may impair reproduction and foetal development. However, further research is required to elucidate the epigenetic profiles associated with mycotoxin and *p,p′*-DDT exposure on placental health and foetal development, and how this predispose to adult dieseases.

Chapter 6

6.1 General thesis conclusion and future perspectives

6.1.1 Relevance of research and general conclusion

Mycotoxins are secondary toxic metabolites naturally produced mainly by *Apergillus*, *Penicillium*, and *Fusarium* species of fungi during their growth on various agricultural produce in the field and/or during storage, and they are recognised as major contaminants of food and feedstuff (Rai et al. 2012). The toxin production potential of these fungal species are mainly influenced by moisture, time, temperature, and food or feed substrates while food and feed contamination by mycotoxins can occur in the field, during harvest, storage, processing and transportation (Pitt et al. 2013). Multiple mycotoxins contaminations of foods and feedstuff have been reported worldwide with developing countries having the highest level of contamination. For instance, Rodrigues and Naehrer (2012) reported that 48% of 7049 feed and feedstuff from Americas, Europe and Asia examined between 2009 and 2011 had two or more mycotoxins, including AFB_1, DON, ZEN and OTA. A different study reported that 75% of wheat samples collected from three European countries had co-occurrence of two or more mycotoxins (Monbaliu et al. 2010). Similarly, multi-mycotoxins have been found in feed, millet, maize and infant foods collected from Burkina Faso and Mozambique (Warth et al., 2012c). In addition, human exposures to multiple mycotoxins have been reported in several population studies using multi-mycotoxin biomarker method (Warth et al. 2013). This multi- mycotoxin contamination of food crops have been attributed to the following reasons: i) most fungal species, especially *Fusarium* can produce several mycotoxins while growing on agricultural products, ii) agricultural crops can be can be contaminated by multiple fungal species which can produce mycotoxins simultaneously and iii) food consumed are usually prepared from a combination of different sources which have been solely contaminated by single or multiple mycotoxins. This evidence underscores the importance of testing the toxic effects of mycotoxin mixtures (Alassane-Kpembi et al., 2015). However, there is still limited information available on the effect of either single or combined mycotoxins on reproduction.

Pesticides of different chemical categories, including the organochlorides, organophosphates, carbamates and pyrethroids are also widely studied for their

endocrine disrupting activity (Wielogorska et al., 2015). Among them, the organochlorides are of significant importance due to their persistent nature and long half-life which allows them to accumulate in the environment and biological systems in wildlife and human beings resulting to various health effects such as infertility, cancer, spontaneous abortion and other reproductive health disorders (ATSDR, 2002). Although banned in many countries, the organochloride pesticide *p,p'*-DDT and its major metabolite *p,p'*-DDE are still found in the food chain and environment, and human exposure leads to their accumulation in adipose tissues (Mrema et al., 2012). Apart from single contamination, pesticide and mycotoxin co-contamination of agricultural products have been reported (Musaiger et al., 2008; Romero-González et al., 2011). Despite the fact that humans and animals alike can be exposed to a range of mixtures of chemical contaminants (including mycotoxins and pesticides), most toxicological studies have only taken into account the effects of exposure to individual contaminant. In addition, they are often 'boxed' to their respective contaminant group (mycotoxins or pesticides), with limited studies investigating mixtures of chemical contaminants co-occurring together spanning different chemical groups groups. Very limited data is available on the combined toxic effects or the 'cocktail effect' of exposure to mycotoxins and/or persistent organochloride pesticides, thus the ability to accurately assess the risks to reproductive health of individual and combined exposure is currently inadequate. In this present study, several methods and different cell lines were applied to evaluate the effect of individual and binary mixtures of mycotoxins and persistent organochloride pesticides on reproduction. This is the first report of a comprehensive assessment of the effects of individual and mixtures of mycotoxins and the pesticides *p,p'*-DDT and *p,p'*-DDE, individually or in combination, on reproductive health and development.

Mycotoxins and persistent organochloride pesticides, either in single or in binary combination, had differential effects on the different parameters evaluated in different cell line - MMV-Luc, MA-10 and BeWo cells. ZEN and its metabolite α-ZOL had oestrogenic effects in MMV-Luc cell line with α-ZOL showing higher oestrogenic potency. Co-incubation of either ZEN or α-ZOL with concentration of E2 equivalent to that found in humans before puberty (0.05 nM), modulated the ER response induced by E2 alone whereas their combination with concentration of E2 equivalent to the serum

concentration in females after pubertal stage (10 nM), decreased ER activation producing a partial agonist effects on ER response. In MA-10 Leydig cells, ZEN, α-ZOL and β-ZOL stimulated cell proliferation at low doses and inhibited cell proliferation at high concentrations which is in accordance of their oestrogenic effects at low doses and ability to induce apoptosis at higher concentrations (Dees tal., 1997; Ahamed et al., 2001). However, only α-ZOL increased cell proliferation in BeWo cells which is as a result of its stronger oestrogenic effects compared to ZEN and β-ZOL. At high concentrations, the three mycotoxins reduced the cell viability of BeWo cells, especially at 64 µM. ZEN, α-ZOL and β-ZOL also significantly increased P4 secretion in treated MA-10 cells compared to vehicle control, but inhibited testosterone levels in the same cells. In BeWo cells, ZEN and α-ZOL had no effect on P4 production whereas β-ZOL significantly increased P4 production. All the three compounds significantly induced E2 levels in BeWo cells and inhibited β-hCG secretion in BeWo cells. The increase of P4 level and reduction of testosterone level in MA-10 cells, and induction of E2 in BeWo cells treated with ZEN and its metabolites could be as a result of the modulation of CYP11A and CYP19A1 activity in these cells. The steroid hormone production is dependent on cAMP synthesis and the activity of steroidogenic enzymes such as CYP11A and CYP19A1 (Hilscherova et al., 2004). Therefore, the use of assays incorporating the activity and gene expression of steroidogenic enzymes as well as complete steroid hormone profiling would provide better understanding of the effects of these compounds on the seteroidogenic pathway.

DON and OTA had no oestrogenic effects in the MMV-Luc reporter gene assay. In both MA-10 and BeWo cell lines, DON and OTA were strongly cytotoxic with IC_{50} values ranging from 13.39 ± 1.12 µM to 21.97 ± 5.25 µM and from 10.21 ± 0.21 µM to 28.50 ± 2.63, respectively in both cell lines. OTA caused induction of P4 and testosterone secretion in MA-10 cells whereas DON decreased both P4 and testosterone levels in the same cell line. However, both DON and OTA inhibited E2, P4 and β-hCG levels in BeWo cell line. It was demonstrated that *p,p'*-DDT and *p,p'*-DDE were weakly oestrogenic in the MMV-Luc cell line, simillar to previous reports (Kuiper et al., 1998; Li et al., 2008; Brennan et al., 2016). In the cytoxicity assay, *p,p'*-DDT and *p,p'*-DDE were not strongly cytooxic to both MA-10 and BeWo cell lines. At the hormonal level, *p,p'*-DDT significantly induced P4 secretion in MA-10 cells but had no significant

effect on testosterone production. In the otherhand, *p,p′*-DDT inhibited both P4 and β-hCG level, but no significant effect on E2 secretion in BeWo cells. A major observation of this study was that all tested compounds strongly inhibited β-hCG production in BeWo cells. Low β-hCG levels during early pregnancy have been linked to miscarriage and implantation failure whereas high β-hCG concentration in late pregnancy is associated with preeclampsia (Paulesu et al., 2018). In addition, impairment of placental endocrine and metabolic function has been associated with pre-eclampsia, intrauterine growth restriction, preterm birth, and low birth weight (Cartwright et al., 2010). Therefore, the results of this study indicate the possibility of adverse pregnancy outcome in women exposed to these chemicals used in this study before or during pregnancy.

Binary combinations of mycotoxins and pesticides have differential effects on E2-mediated ER transcriptional response in MMV-Luc cell reporter gene assay. For instance, co-treatment of either ZEN + α-ZOL, ZEN + *p,p′*-DDT or ZEN + *p,p′*-DDE with E2 (0.05 nM) enhanced the transcriptional response mediated by E2 whereas at 10 nM of E2, these binary combinations had inhibitory effect on E2-mediated ER transcriptional response. Similarly, co-exposure of either α-ZOL + *p,p′*-DDT or α-ZOL + *p,p′*-DDE modulated the ER response at low dose of E2 (0.05 nM), but had inhibitory effect at high dose of (10 nM) with the highest combintions reducing E2-mediated ER response by over 70%. Most importantly, co-incubation of *p,p′*-DDT + *p,p′*-DDE (50 µM each) completely inhibited the ER response mediated by both 0.05 nM or 10 nM of E2. In MA-10 cells, co-treatment of mycotoxins with *p,p′*-DDT enhanced the cytotoxic effects of each mycotoxin, whereas co-exposure with *p,p′*-DDE had no significant difference in cytoxicity compared to each of the mycotoxins, alone. In addition, OTA enhanced the cytotoxic effects of ZEN, α-ZOL and β-ZOL in MA-10 Cells. However, co-treatment of either *p,p′*-DDT or *p,p′*-DDE with ZEN, α-ZOL or β-ZOL enhanced their cytotoxic effects on BeWo cell line. Also worthy of note, mixtures of OTA + ZEN, OTA + α-ZOL, OTA + β-ZOL, ZEN + α-ZOL, ZEN + β-ZOL and α-ZOL + β-ZOL produced synergistic cytotoxic effects at low doses in BeWo cells. In general, most of the mycotoxin and pesticide mixtures produced synergistic or additive cytotoxicity at lower concentrations, but showed antagonistic cytotoxic effects at concentrations above 16 µM in both MA-10 and BeWo cell lines. At the hormonal level, binary mixtures of

p,p'-DDT with either ZEN, α-ZOL or β-ZOL increased P4 hormone level in MA-10 cells, but these were not significantly different from the effect of each compound alone, but significantly reduced testosterone secretions in the same cell line. In contrast, co-treatment of α-ZOL with β-ZOL enhanced both P4 and testosterone secretion in MA-10 cells. In BeWo cells, binary mixtures of *p,p'*-DDT with either ZEN, α-ZOL or β-ZOL had no effect on both P4 and E2 hormone levels, but upregulated the secretion of β-hCG levels. In addition, ZEN + α-ZOL or α-ZOL + β-ZOL had no effect on P4 hormone production in BeWo cells but synergistically increased E2 hormone level in BeWo cells.

To further understand the molecular mechanisms through which EDCs could affect reproduction, this study examined the effects of exposure to ZEN and *p,p'*-DDT, individually and in combination, on the mRNA expression of placental steroidogenic and imprinted genes as well as genes involved in DNA methylation. Most importantly, ZEN and *p,p'*-DDT, alone or in combination differentially upregulated the expression of genes of the IGF-axis (*IGF2BP1* and *IGF2R*), genomic imprinting (*PHLDA2* and *MEG3*) and DNA methylation (*MTHFR* and *MDB2*). Taken together, it can be deduced that mycotoxins and *p,p'*-DDT pesticide affect placental endocrine and metabolic function through different mechanisms. An elevated placental expression of the imprinted gene *PHLDA2* has been implicated in low birth weight and intrauterine growth restriction (McMinn et al., 2006; Apostolidou et al., 2007) as well as in spontaneous abortion and *in utero* foetal death (Dória et al., 2010) in humans. Elevated expression of *IGFBP1* has been reported in another study on growth restricted placentas (Okamoto *et al.* 2006). Also, it is well established that IGFBPs inhibit the biological function of IGFs by competing with the IGF receptors for the peptide. Therefore, decreased availability of IGFs and increased expression of IGFBPs can result in impaired embryo and foetal development and consequently, intrauterine growth restricted (IUGR) pregnancies (Carter *et al.* 2005). However, additional research is needed to further explore the role epigenetics play on reproduction after exposure to these environmental and food-borne toxins.

6.1.2 Future perspectives

This study has provided a comprehensive assessment of the effects of mycoxins and persistent organochloride pesticides on reproduction using biologically and environmentally relevant concentrations, relevant *in vitro* methods and suitable cell lines. Overall, the results indicate that these toxins can have adverse effects on reproduction by affecting reproductive cell morphology, perturbation of nuclear receptor response and hormone production as well as the dysregulation of genes involved in steroidogenesis and epigenetics. From the result of this study, it can be stated that the effects of mycotoxins and *p,p'*-DDT pesticide are multifaceted and these effects are influenced by the cell lines employed in the study.

The recommendations for further work include and are not limited to:

1. More research is required to explore the reproductive health effects using cell lines or tissue explants from other reproductive tissues such as the endometrium and ovary as well as co-culture methods to enable a more accurate and relevant conclusion when results are extrapolated to *in vivo* scenario.

2. The endocrine disrupting effects of these mycotoxins and pesticides, either in single or binary mixtures, on the level of nuclear receptor transcriptional activation should further be explored on pregnane X receptor, constitutive androstane receptor, progesterone receptor, androgen receptor and aryl hydrocarbon receptor.

3. Future studies should examine the genome-wide DNA methylation and gene expression profile for epigenetic alterations associated with exposure to mycotoxins and persistent organochloride pesticides, either in single or mixtures in order to understand their potential transgenerational toxicity.

4. Human epidemiological studies would certainly provide stronger evidence for establishing a causal association**.**

5. A complete hormonal profiling of the placenta and Leydig cells using LC-MS/MS will provide further information in understanding perturbations of hormone production by EDCs.

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Appendices

Appendix 1: The concentrations of mycotoxins and pesticides used in the study

Toxins	Concentrations (µM)	Concentrations (ng/mL)
p,p'-DDT	0.1	35.449
	1	354.49
	8	2,835.92
	16	5,671.84
	32	11,343.68
	64	22,687.36
p,p'-DDE	0.1	31.803
	1	318.03
	8	2,544.24
	16	5,088.48
	32	10,176.96
	64	20,353.92
DON	0.1	29.632
	1	296.32
	8	2,370.56
	16	4,741.12
	32	9,842.24
	64	18,964.48
OTA	0.1	40.381
	1	403.81
	8	3,230.48
	16	6,460.96
	32	12,921.92
	64	25,843.84
AFB_1	0.1	31.227
	1	312.27
	8	2,498.16
	16	4,996.36
	32	9,992.64
	64	19,985.28
ZEN	0.1	31.836
	1	318.36
	8	2,546.88
	16	5,093.76
	32	10,187.52
	64	20,375.04
α-/β-ZOL	0.1	32.038
	1	320.38
	8	2,563.04
	16	5,126.08
	32	10,252.16
	64	20,504.32

APPENDIX 2

Appendix 2: Details of the primers of housekeeping genes and target genes

S/No	Symbol	Name	Accession	Size	Forward Primer	Reverse Primer
1	ABCC1	ATP-binding cassette, sub-family C (CFTR/MRP), member 1	NM_004996	209	TGTCAACCTCATGTCTGTGG	CCACCTGATACGTCTTGGTC
2	ABCC2	ATP-binding cassette, sub-family C (CFTR/MRP), member 2	NM_000392	185	TCCTGGTGGATAGCAACAAT	AATGGCAGATGTGTCCAAGT
3	STAR	steroidogenic acute regulatory protein	NM_000349	250	GGCTACTCAGCATCGACCTC	CATCCCACTGTCACCAGATG
4	CYP11A1	cytochrome P450, family 11, subfamily A, polypeptide 1	NM_000781	150	ACCCAGCAGTGATCAGAGAG	CACTGAGAACCCATTCAACC
5	CYP17A1	cytochrome P450, family 17, subfamily A, polypeptide 1	NM_000102	233	CCCTGATCCTAACTGTGTGG	GGCACAGCTTTGACTGAAAT
6	CYP19A1	cytochrome P450, family 19, subfamily A, polypeptide 1	NM_000103	242	CAGAGGCCAAGAGTTTGAGG	ACACTAGCAGGTGGGTTTGG
7	HSD3B1	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1	NM_000862	250	AGCTTCCTACTCAGGCCAAT	TACCCACATGCACATCTCTG
8	HSD17B1	hydroxysteroid (17-beta) dehydrogenase 1	NM_000413	193	ATGAGCTAGGAACCCTGGAC	AGGCTTTACAAGCAGGTGTG
9	HMGCR	3-hydroxy-3-methylglutaryl-CoA reductase	NM_000859	231	CTTGGTTTTTGGCTCTTTCA	GTCAATTGCACTGATCACCA
10	ESR1	estrogen receptor 1 (ER alpha)	NM_000125	211	GAGGATTCCCGTAGCTCTTC	CCCTTGACCTAGCTTTCTCC
11	ESR2	estrogen receptor 2 (ER beta)	NM_001437	151	GGCAGAGGACAGTAAAAGCA	GGACCACACAGCAGAAAGAT
12	PGR	Progesterone receptor	NM_000926	200	ACAGGAAAGCAGCACAATTC	CCAAAACCCTTCTGGAAAAT
13	AR	Androgen receptor	NM_000044	155	CGGAAGCTGAAGAAACTTGG	ATGGCTTCCAGGACATTCAG

14	AHR	aryl hydrocarbon receptor	NM_001621	178	ACGGATGAAATCCTGACGTA	GTTGCTGTGGCTCCACTACT
15	PPARG	peroxisome proliferator-activated receptor gamma	NM_138712	200	CCCAAGTTTGAGTTTGCTGT	AACAGCTGTGAGGACTCAGG
16	LHCGR	luteinizing hormone/choriogonadotropin receptor	NM_000233	157	TCAATTCTTGTGCCAATCCA	CCATTTTTGCAGTTGGAGGT
17	CDH1	cadherin 1, type 1, E-cadherin (epithelial)	NM_004360	423	TGCTCTTGCTGTTTCTTCGG	TGCCCCATTCGTTCAAGTAG
18	GPER1	G protein-coupled estrogen receptor 1	NM_001505	174	ATTCGCTGAAGTTCCCTTCT	TGGCCATTTGAATTTTCACT
19	IGF2	insulin-like growth factor 2	NM_000612	355	AGACACCAATGGGAATCCCAA	TATTGGAAGAACTTGCCCACG
20	IGF2AS	IGF2 antisense RNA	NR_028044	209	GTTTGCCTAGAAAGGGCTTC	CCCTCCTTATCTCCTTCCAA
21	INS	insulin	NM_000207	210	CTCACACCTGGTGGAAGCTC	AGGGAGCAGATGCTGGTACA
22	INSR	insulin receptor	NM_000208	217	CAGCTGAGCTAGAAGCCAAC	TGAGTGATGGTGAGGTTGTG
23	IGF1	insulin-like growth factor 1 (somatomedin C)	NM_000618	235	CCCCACTCACCAACTCATAG	GGTATTTGGGGCCTTTATGT
24	IGF1R	insulin-like growth factor 1 receptor	NM_000875	205	TTAAGAACCAGTGGCGAAAG	GGAGCACTCACTTCTCCAAA
25	IGF2R	insulin-like growth factor 2 receptor	NM_000876	203	CATGGGAAGCTGTTGATACC	CTGGTCACAGCTCACTGTTG
26	IGF2BP1	insulin-like growth factor 2 mRNA binding protein 1	NM_006546	237	CAACCCAGACATACCCTCTG	AAGAAACAGGCAAACAGTCG
27	IGF2BP2	insulin-like growth factor 2 mRNA binding protein 2	NM_006548	215	TGTGCCAGTGCTGAGATAGA	TATCCGGAGTGGGTAGTGAA
28	IGF2BP3	insulin-like growth factor 2 mRNA binding protein 3	NM_006547	215	CACTTCTATGCTTGCCAGGT	GCATGTGATTCTGGATAGGG
29	PHLDA2	pleckstrin homology-like domain, family A, member 2	NM_003311	145	GCCAGTTTGCTTTTCTACCA	TATTCATTCAAAGCCGGTTC

30	MEG3	maternally expressed 3 (non-protein coding)	NR_002766	176	ATTAAGCCCTGACCTTTGCT	AAAGGGATCCTTCCATTCAG
31	DNMT1	DNA (cytosine-5-)-methyltransferase 1	NM_001379	293	CGAGTTGGTGATGGTGTGT	GTGGACTTGTGGGTGTTCTC
32	DNMT3A	DNA (cytosine-5-)-methyltransferase 3 alpha	NM_175629	287	CTGTGATGATTGATGCCAAA	GACTGGGAAACCAAATACCC
33	DNMT3B	DNA (cytosine-5-)-methyltransferase 3 beta	NM_006892	265	ATAAGACACCCCCTCAAACC	TTCCCGTTCTCCCTAAAAAC
34	TET3	tet methylcytosine dioxygenase 3	NM_001287491	175	GAACCACTTCAGCTCCTTCA	GAGAGCGTACTTGGAGTGGA
35	MTHFR	methylenetetrahydrofolate reductase (NAD(P)H)	NM_005957	168	CAGCTGAAACATTCCGAAGA	CCTGGGCTCAGAGTAAGGAG
36	BHMT	betaine--homocysteine S-methyltransferase	NM_001713	208	CGACTGAAAGCTCACCTGAT	TGATGTGGTAGGGCTCAAAT
37	MBD2	methyl-CpG binding domain protein 2	NM_003927	216	TCTGGACTTTCAGTGGCTTC	ATGGCAAAAATCAGGATTCA
38	LEP	leptin	NM_000230	179	GAAGGTCACTCTTCCTGCAA	TTGGTTTCAAGCAAAAGGAG
39	DYSF	dysferlin	NM_001130987	240	GCAAGAATTGTGGGAAGCTA	AAAACTTGGGGTAGGTGAGG
40	ERVW-1	endogenous retrovirus group W, member 1	AF072506	174	GGGTTCCATGGTTCTCTTCT	TGGTGAACCACTTCCAAGAT
41	ERVFRD-1	endogenous retrovirus group FRD, member 1	NM_207582	195	CTCATTCTCACGCCTTCACT	TAATTCCGCCTCTATGCTTG
42	VEGFA	Vascular endothelial growth factor A	NM_001025366	231	AGACACACCCACCCACATAC	TGCCAGAGTCTCTCATCTCC
43	MMP2	Matrix metalloproteinase 2	NM_004530	153	TTGACGGTAAGGACGGACTC	ACTTGCAGTACTCCCCATCG
44	ZFP57	Zinc finger protein 57	NM_001109809	221	GTTTGTTCACAATCCCAAGC	AGCTCTTCCCACACACAGAG
45	RPLP0	ribosomal protein, large, P0	NM_003406	183	AGCTTTTGATGAAGCCATTG	GAGGCAGACAAAAGTTGGAA

46	HPRT1	Hypoxanthine guanine phosphoribosyl transferase 1	NM_000194	94	TGACACTGGCAAAACAATGCA	GGTCCTTTTCACCAGCAAGCT
47	PGK1	Phosphoglycerate kinase 1	NM_000291	198	CTGTGGGGGTATTTGAATGG	CTTCCAGGAGCTCCAAACTG
48	GAPDH	glyceraldehyde-3-phosphate dehydrogenase	NM_002046	238	GAGTCAACGGATTTGGTCGT	TTGATTTTGGAGGGATCTCG
49	YWHAZ	Tyrosine 3-monoxygensase/tryptophan 5-monoxygenase activation protein, zeta	NM_001002	236	AACCCAGCTCTGGAGAAACT	CCCCTGGAGATTTTAGTGGT
50	ACTB	actin, beta	NM_001101	233	GGACTTCGAGCAAGAGATGG	AGCACTGTGTTGGCGTACAG